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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/82, 15/52, C12P 17/06, 23/00, 7/64, C11B 1/00, A01H 5/00 — A1

(11) International Publication Number: **WO 98/06862**

(43) International Publication Date: 19 February 1998 (19.02.98)

(21) International Application Number: PCT/US97/14035

(22) International Filing Date: 8 August 1997 (08.08.97)

(30) Priority Data:
60/024,145 9 August 1996 (09.08.96) US

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(81) Designated States: AL, AU, BA, BB, BG, BR, CA, CN, CU, CZ, EE, GE, HU, IL, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, SL, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHODS FOR PRODUCING CAROTENOID COMPOUNDS AND SPECIALITY OILS IN PLANT SEEDS

(57) Abstract

Methods are provided for producing plants and seeds having altered carotenoid, fatty acid and tocopherol compositions. The methods find particular use in increasing the carotenoid levels in oilseed plants and in providing desirable high oleic acid seed oils.

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METHODS FOR PRODUCING CAROTENOID COMPOUNDS AND SPECIALITY OILS IN PLANT SEEDS

FIELD OF THE INVENTION

The invention relates to genetic modification of plants, plant cells and seeds, particularly altering carotenoid biosynthesis and fatty acid composition.

BACKGROUND OF THE INVENTION

Carotenoids are pigments with a variety of applications. They are yellow-orange-red lipids which are present in green plants, some molds, yeast and bacteria. Carotenoid hydrocarbons are referred to as carotenes, whereas oxygenated derivatives are referred to as xanthophylls. The carotenoids are part of the larger isoprenoid biosynthesis pathway which, in addition to carotenoids, produces such compounds as chlorophyll and tocopherols, Vitamin E active agents. The carotenoid pathway in plants produces carotenes, such as α- and β-carotene, and lycopene, and xanthophylls, such as lutein.

The biosynthesis of carotenoids involves the condensation of two molecules of the C_{20} precursor geranyl PP_i to yield the first C_{40} hydrocarbon phytoene. In a series of sequential desaturations, phytoene yields lycopene. Lycopene is the precursor of the cyclic carotenes, ß-carotene and α-carotene. The xanthophylls, zeaxanthin and lutein are formed by hydroxylation of ß-carotene and α-carotene, respectively.

ß-carotene, a carotene whose color is in the spectrum ranging from yellow to orange, is present in a large amount in the roots of carrots and in green leaves of plants. ß-carotene is useful as a coloring material and also as a precursor of vitamin A in mammals. Current methods for commercial production of ß-carotene include isolation from carrots, chemical synthesis, and microbial production.

A number of crop plants and a single oilseed crop are known to have substantial levels of carotenoids, and consumption of such natural sources of carotenoids have been indicated as providing various beneficial health effects. The below table provides levels of carotenoids that have been reported for various plant species.

CAROTENOID CONTENTS OF VARIOUS CROPS
(µg/g)

Crop	Beta-Carotene	Alpha-Carotene	Lycopene	Lutein	Total
Carrots	30-110	10-40	0-0.5	0-2	65-120
Pepper (gr)	2	-	-	2	8
Pepper (red)	15	1	-	-	200
Pumpkin	16	0.3	tr	26	100
Tomato	3-6	-	85	-	98
Watemelon	1	tr	19	-	25
Marigold petals	5	4	-	1350	1500
Red palm oil	256	201	8	-	545

The pathway for biosynthesis of the carotenoids has been studied in a variety of organisms and the biosynthetic pathway has been elucidated in organisms ranging from bacteria to higher plants. See, for example, Britton, G. (1988) *Biosynthesis of carotenoids*, p. 133-182, In T.W. Goodwin (ed.), Plant pigments, 1988. Academic Press, Inc. (London), Ltd., London. Carotenoid biosynthesis genes have also been cloned from a variety of organisms including *Erwinia uredovora* (Misawa *et al.* (1990) *J. Bacteriol. 172*:6704-6712; *Erwinia herbicola* (Application WO 91/13078, Armstrong *et al.* (1990) *Proc. Natl. Acad. Sci., USA 87*:9975-9979); *R. capsulatus* (Armstrong *et al.* (1989) *Mol. Gen. Genet. 216*:254-268, Romer *et al.* (1993) *Biochem. Biophys. Res. Commun. 196*:1414-1421); *Thermus thermophilus* (Hoshino *et al.* (1993) *Appl. Environ. Microbiol. 59*:3150-3153); the cyanobacterium *Synechococcus sp.* (Genbank accession number X63873). See also, application WO 96/13149 and the references cited therein.

While the genes have been elucidated, little is known about the use of the genes in plants. Investigations have shown that over expression or inhibition of expression of the plant phytoene synthase (Psy1) gene in transgenic plants can alter carotenoid levels in fruits. See, Bird *et al.* (1991) *Biotechnology* 9:635-639; Bramley *et al.* (1992) *Plant J.* 2:343-349; and Fray and Grierson (1993) *Plant Mol. Biol.* 22:589-602. Further, as reported by Fray *et al.* (1995) *The Plant Journal* 8:693-701, constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway.

Application WO 96/13149 reports on enhancing carotenoid accumulation in storage organs such as tubers and roots of genetically engineered plants. The application is directed towards enhancing colored native carotenoid production in specific, predetermined non-photosynthetic storage organs. The examples of the application are drawn to increasing colored carotenoids in transformed carrot roots and in orange flesh potato tubers. Both of these tissues are vegetative tissues, not seeds, and natively have a high level of carotenoids.

Carotenoids are useful in a variety of applications. Generally, carotenoids are useful as supplements, particularly vitamin supplements, as vegetable oil based food products and food ingredents, as feed additives in aminal feeds and as colorants. Specifically, phytoene finds use in treating skin disorders. See, for example, U.S. Patent No. 4,642,318. Lycopene, α- and β-carotene are used as food coloring agents. Consumption of β-carotene and lycopene has also been implicated as having preventative effects against certain kinds of cancers. In addition, lutein consumption has been associated with prevention of macular degeneration of the eye.

Plant oils are useful in a variety of industrial and edible applications. Novel vegetable oils compositions and/or improved means to obtain oils compositions, from biosynthetic or natural plant sources are needed. Depending upon the intended oil use, various different fatty acid compositions are desired. The demand for modified oils

with specific fatty acid compositions is great, particularly for oils high in oleic acid. See, Haumann, B. F. (1996) *INFORM* 7:320-334. As reported by Haumann, the ideal frying oil would be a low-saturate, high oleic and low linolenic oil. Furthermore, studies in recent years have established the value of monounsaturated fatty acids as a dietary constituent.

Attempts have been made over the years to improve the fatty acid profiles of particular oils. For example, the oxidative stability of vegetable oil is related to the number of double bonds in its fatty acids. That is, molecules with several double bonds are recognized to be more unstable. Thus, scientists have attempted to reduce the content of α-linolenic acid in order to improve shelf life and oxidative stability, particularly under heat.

It is apparent that there is needed a method for producing significant levels of carotenoid compounds in crop plants and particularly in plant seeds. It would additionally be beneficial to alter the fatty acid content of the plants and seeds. Such altered seed products would be useful nutritionally as well as provide a source for producing more stable oils. There is no report of methods to substantially altering the levels and composition of carotenoids produced in a plant seed, particularly with respect to increasing the level of production of carotenoids. There is therefore needed, a useful method for altering carotenoid levels in plants, particularly seeds, and for producing oils with modified carotenoid composition and/or content.

SUMMARY OF THE INVENTION

Transformed plants, plant cells and seeds having altered carotenoid levels and/or modified fatty acid compositions are provided. The plants, plant cells and seeds are transformed with at least one carotenoid biosynthesis gene. Methods for making and using the transformed compositions of the invention are also provided. Methods find use in altering carotenoid levels in plants, particularly seeds, as well as increasing particular compounds for molecular farming, such as for production of particular carotenoids and tocopherols. At the same time, the transformed compositions, particularly seeds, provide a source of modified oils, which oils may be extracted from the seeds in order to provide an oil product comprising a natural source of various carotenoids and carotenoid mixtures. In a particular aspect of the present invention transformed seed can provide a source for particular carotenoid compounds and/or for modified speciality oils having altered carotenoid or tocopherol compostions and/or altered fatty acid composition, particularly having increased levels of oleic acid and decreased levels of linoleic and linolenic acids.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the nucleotide sequence of the SSU/*crt*B fusion sequence.

Figure 2 presents constructs for expression of carotenoid biosynthesis genes in plant seeds. Figure 2A shows plasmid pCGN3390 which contains the napin promoter operably linked to the SSU/*crtB* sequence. Figure 2B shows plasmid pCGN3392which contains the napin promoter operably linked to the SSU/*crtE* sequence. Figure 2C shows plasmid pCGN9010 which contains the napin promoter operably linked to the SSU/*crtI* sequence. Figure 2D shows plasmid pCGN9009 which contains the napin promoter operably linked to the SSU/*crtB* sequence and the napin promoter operably linked to the SSU/*crtI* sequence. Figure 2E shows plasmid pCGN9002 which contains the napin promoter operably linked to the SSU/*crtB*

sequence and the napin promoter operably linked to an antisense epsilon cyclase sequence. Figure 2F shows plasmid pCGN9017 which contains the napin promoter operably linked to the SSU/*crtB* sequence and the napin promoter operably linked to an antisense beta cyclase sequence.

Figure 3 shows the results of analyses of saponified samples for control seeds.

Figure 4 shows the results of analyses of saponified samples for pCGN3390 transformed seeds.

Figure 5 shows a graph of the fatty acid analysis in pCGN3390 transformed seeds and demonstrates that the increase in 18:1 fatty acids correlates with a decrease in 18:2 and 18:3.

Figure 6 shows a graph of the fatty acid analysis in pCGN3390 transformed seeds and demonstrates that the increase in 18:1 correlates with an increase in both 18:0 and 20:0, but little effect is seen in 16:0.

Figure 7 shows a graph of the fatty acid analysis in pCGN3390 transformed seeds and demonstrates the increase in 18:0 correlates well with an increase in 20:0.

Figure 8 shows a carotenoid biosynthesis pathway.

Figure 9 provides sequence of *B. napus* epsilon cyclase cDNA clone 9-4.

Figure10 provides sequence of *B. napus* epsilon cyclase cDNA clone 7-6.

Figure 11 provides sequence of a *B. napus* beta cyclase cDNA clone.

Figure 12 provides T2 seed analysis of 3390 transformed *Brassica napus* plants.

Figure 13 provides T3 seed analysis of 3390 transformed *Brassica napus* plants.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the subject invention, methods for increasing production of carotenoid compounds as well as for altering fatty acid compositions in a plant, particularly in plant seeds are provided. The method involves transforming a plant cell with at least one carotenoid biosynthesis gene. This has the effect of altering carotenoid biosynthesis particularly increasing the production of downstream products, as well as providing novel seed oils having desirable fatty acid compositions. A second gene can then be utilized to shunt the metabolic activity to the production of particular carotenoid compounds or to further alter the fatty acid composition.

Surprisingly it has been found that transformation of a plant with an early carotenoid biosynthesis gene leads to a significant increase in the flux through the carotenoid pathway resulting in an increase in particular carotenoids. That is, there is an increase in the metabolic activity that can be further manipulated for the production of specific carotenoids. In addition, the transformed seeds may demonstrate altered fatty acid compositions as the result of the carotenoid gene expression, such as seen with the seeds described herein from plants transformed with a phytoene synthase gene.

Thus, using the methods of the invention seeds are provided which produce high levels of a particular carotenoid and/or produce speciality oils having a desired fatty acid composition. In oilseed *Brassica*, for example, transformation with an early carotenoid biosynthesis gene leads to seeds having a significant increase in the production of α-carotene, β-carotene and lutein. In addition, the *Brassica* seeds demonstrate an altered fatty acid composition and yield a vegetable oil which has increased oleic acid content and decreased linoleic and linolenic acid content. Thus, the transformed seed can provide a source of carotenoid products as well as modified seed oil. In this manner, modified speciality oils can be produced and new sources of carotenoids for extraction and purification are provided.

The oils of the present invention also provide a substantial improvement with

respect to stability as compared to two other major plant sources of carotenoids, marigold petals and red palm oil (mesocarp). Although instability is observed in seeds stored in air at room temperature as demonstrated by loss of approximately 20-30% of total carotenoids after 4 weeks of storage, the loss after 1-2 weeks is only 10%. Palm mesocarp, by contrast, must be processed within a day or two of harvest in order to avoid major losses of carotenoid. Furthermore, the carotenoid decomposition in the seeds of the present invention may be reduced significantly by storage of the seeds under nitrogen.

For the production of a seed having an increase in carotenoid biosynthesis, transformation of the plant with an early carotenoid biosynthesis gene is sufficient. By early carotenoid biosynthesis gene is intended geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and isopentenyl diphosphate (IPP) isomerase. A variety of sources are available for the early carotenoid biosynthesis genes and for the most part, a gene from any source can be utilized. However, it is recognized that because of co-suppression, the use of a plant gene native to the target host plant may not be desirable where increased expression of a particular enzyme is desired.

A number of early carotenoid biosynthesis genes have been isolated and are available for use in the methods of the present invention. See, for example:

IPP isomerase has been isolated from: *R. Capsulatus* (Hahn *et al.* (1996) *J. Bacteriol. 178*:619-624 and the references cited therein). GenBank Accession Nos. U48963 and X82627, *Clarkia xantiana* GenBank Accession No. U48962, *Arabidopsis thaliana* GenBank Accession No. U48961, *Schizosaccharmoyces pombe* GenBank Accession No. U21154, human GenBank Accession No. X17025, *Kluyveromyces lactis* GenBank Accession No. X14230;

geranylgeranyl pyrophosphate synthase from *E. Uredovora* Misawa *et al.* (1990) *J. Bacteriol. 172*:6704-6712 and Application WO 91/13078; and from plant sources, including white lupin (Aitken *et al.* (1995) *Plant Phys.108*:837-838), bell

pepper (Badillo *et al.* (1995) *Plant Mol. Biol.* 27:425-428) and *Arabidopsis* (Scolnik and Bartely (1994) *Plant Physiol. 104*:1469-1470; Zhu *et al.* (1997) *Plant Cell Physiol. 38*:357-361).

phytoene synthase from a number of sources including *E. Uredovora*, *Rhodobacter capsulatus*, and plants Misawa *et al.* (1990) *J. Bacteriol. 172*:6704-6712, GenBank Accession No. D90087, Application WO 91/13078, Armstrong *et al.* (1989) *Mol. Gen. Genet. 216*:254-268, Armstrong, G. A. "Genetic Analysis and regulation of carotenoid biosynthesis. *In* R. C. Blankenship, M. T. Madigan, and C. E. Bauer (ed.). *Anoxygenic photosynthetic bacteria; advances in photosynthesis.* Kluwer Academic Publishers, Dordrecht, The Netherlands, Armstrong *et al.* (1990) *Proc. Natl. Acad. Sci USA 87*:9975-9979, Armstrong *et al.* (1993) *Methods Enzymol. 214*:297-311, Bartley and Scolnik (1993) *J. Biol. Chem. 268*:27518-27521, Bartley *et al.* (1992) *J. Biol. Chem. 267*:5036-5039, Bramley *et al.* (1992) *Plant J. 2*:291-343, Ray *et al.* (1992) *Plant Mol. Biol. 19*:401-404, Ray *et al.* (1987) *Nucleic Acids Res. 15*:10587, Romer *et al.* (1994) *Biochem. Biophys. Res. Commun. 196*:1414-1421, Karvouni *et al.* (1995) Plant *Molecular Biology* 27:1153-1162, GenBank Accession Nos. U32636, Z37543, L37405, X95596, D58420, U32636, Z37543, X78814, X82458, S71770, L27652, L23424, X68017, L25812, M87280, M38424, X69172, X63873, and X60441, Armstrong, G. A. (1994) *J. Bacteriol. 176*:4795-4802 and the references cited therein; and,

phytoene desaturase from bacterial sources including *E. uredovora* Misawa *et al.* (1990) *J. Bacteriol. 172*:6704-6712, and Application WO 91/13078 (GenBank Accession Nos. L37405, X95596, D58420, X82458, S71770, and M87280); and from plant sources, including maize (Li *et al.* (1996) *Plant Mol. Biol. 30*:269-279), tomato (Pecker *et al.* (1992) *Proc. Nat. Acad. Sci. 89*:4962-4966 and Aracri *et al.* (1994) *Plant Physiol. 106*:789), and *Capisum annuum* (bell beppers) (Hugueney *et al.* (1992) *J. Biochem. 209*: 399-407), GenBank Accession Nos. U37285, X59948, X78271, and X68058).

See, generally, Misawa *et al.* (1990) *J. of Bacteriology* 172:6704-6712, E.P. 0393690 B1, U.S. Patent No. 5,429,939, Bartley *et al.* (1992) *J. Biol. Chem.* 267:5036-5039, Bird *et al.* (1991) *Biotechnology* 9:635-639, and US Patent No. 5,304,478, which disclosures are herein incorporated by reference.

Transformation with an early carotenoid gene, (referred to also as the primary gene), increases the biosynthetic activity of the carotenoid pathway, and can lead to increased production of particular carotenoids such as for example, α- and β-carotene. As described in more detail in the following examples, by expression of a phytoene synthase as the primary gene, large increases in the carotenoid content generally, and particularly in the levels of α- and β-carotene, are obtained in seeds of transformed plants. Oil comprising the carotenoids so produced may be extracted from the seeds to provide a valuable source of α- and β-carotenes. Such an oil may find use as a food colorant, for example to add color to margarines, or as a food oil. An edible food oil with high α- and β-carotene levels is of interest for prevention of Vitamin A deficiency which can result in night blindness. Thus, production of the transformed plants and extraction of the high α- and β-carotene oil to provide a useful food oil is particularly desirable in regions where night blindness is a widespread problem, such as in India and Asia.

In addition to the high high α- and β-carotene levels, levels of other carotenoids are also increased in the oils exemplified herein. For example, lutein levels are increased in seeds from plants transformed with a phytoene synthase gene, as well as in seeds from plants transformed with a GGPP synthase gene.

Furthermore, additional primary genes may be expressed to provide for even greater flux through the carotenoid pathway. For example, in the oilseed *Brassica* seeds containing the phytoene synthase gene as described herein, increased levels of phytoene are observed. Thus, increasing the expression of phytoene desaturase as well as the phytoene synthase may result in further increases in the levels of

carotenoids, such as α- and β-carotene and lutein, produced. Furthermore, plants expressing both the phytoene synthase and the GGPP synthase genes are desirable and may be produced by crossing the 3390 and 3392 plants comprising these genes which are described herein.

In addition to the production of the carotenoids described herein, once the biosynthetic activity has been increased by expression of the primary carotenoid biosynthesis gene or genes, the pathway can be diverted for the production of specific compounds. The diversion involves the action of at least one second gene of interest, (the secondary gene). The secondary gene can encode an enzyme to force the production of a particular compound or alternatively can encode a gene to stop the pathway for the accumulation of a particular compound. For forcing the production of a particular compound, expression of a carotenoid biosynthesis gene in the pathway for the desired carotenoid compound is used. Genes native or foreign to the target plant host may find use in such methods, including, for example carotenoid biosynthesis genes from sources other than higher plant, such as bacteria, including *Erwinia* and *Rhodobacter* species. For stopping the pathway in order to accumulate a particular carotenoid compound, the secondary gene will provide for inhibition of transcription of a gene native to the target host plant, wherein the enzyme encoded by the inhibited gene is capable of modifying the desired carotenoid compound. Inhibition may be achieved by transcription of the native gene to be inhibited in either the sense (cosuppression) or antisense orientation of the gene.

For example, for alteration of the carotenoid composition towards the accumulation of higher levels of ß-carotene derived carotenoids, such as zeaxanthin, zeaxanthin diglucoside, canthaxanthin, and astaxanthin, inhibition of lycopene epsilon cyclase is desired to prevent accumulation of alpha carotene and it's derivative carotenoids, such as lutein. In conjunction with the inhibition of lycopene epsilon cyclase, increased expression of a secondary gene may be desired for increased

accumulation of a particular beta-carotene derived carotenoid. For example, increased β-carotene hydroxylase expression is useful for production of zeaxanthin, wherease increased β-carotene hydroxylase and keto-introducing enzyme expression is useful for production of astaxanthin. Alternatively, for accumulation of lycopene, inhibition of lycopene beta cyclase or of lycopene epsilon cyclase and lycopene beta cyclase is desired to reduce conversion of lycopene to alpha- and beta-carotene.

Secondary genes of interest in the present application include but are not limited to:

β-carotene hydroxylase or *crt*Z (Hundle *et al.* (1993) *FEBS Lett. 315*:329-334, GenBank Accession No. M87280) for the production of zeaxanthin;

genes encoding keto-introducing enzymes, such as*crt*W (Misawa *et al.* (1995) *J. Bacteriol. 177*:6575-6584, WO 95/18220, WO 96/06172) or ß-C-4-oxygenzse (*crt*O; Harker and Hirschberg (1997) *FEBS Lett. 404*:129-134) for the production of canthaxanthin;

*crt*Z and *crt*W or *crt*O for the production of astaxanthin;

ε-cyclase and ε-hydroxylase for the production of lutein;

ε-hydroxylase and crtZ for the production of lutein and zeaxanthin;

antisense lycopene ε-cyclase (GenBank Accession No. U50738) for increased production of β-carotene;

antisense lycopene ε-cyclase and lycopene β-cyclase (Hugueney *et al.* (1995) *Plant J. 8*:417-424, Cunningham FX Jr (1996) *Plant Cell 8*:1613-1626, Scolnik and Bartley (1995) *Plant Physiol. 108*:1343, GenBank Accession Nos. X86452, L40176, X81787, U50739 and X74599) for the production of lycopene;

antisense plant phytoene desaturase for the production of phytoene; etc.

In this manner, the pathway can be modified for the high production of any particular carotenoid compound of interest. Such compounds include but are not limited to α-cryptoxanthin, β-cryptoxanthin, ζ-carotene, phytofluene, neurosporane,

and the like. Using the methods of the invention, any compound of interest in the carotenoid pathway can be produced at high levels in a seed.

The pathway can also be manipulated to decrease levels of a particular carotenoid by transformation of antisense DNA sequences which prevent the conversion of the presursor compound into the particular carotenoid being regulated.

Secondary genes can also be selected to alter the fatty acid content of the plant for the production of speciality oils. For example, acyl-ACP thioesterase genes having specificity for particular fatty acid chain lengths may be used. See, for example, USPN 5,304,481, USPN 5,455,167, WO 95/13390, WO 94/10288, WO 92/20236, WO 91/16421, WO 97/12047 and WO 96/36719. Other fatty acid biosynthesis genes of interest include, but are not limited to, ß-keto acyl-ACP synthases (USPN 5,510.255), fatty acyl CoA synthases (USPN 5,455,947), fatty acyl reductases (USPN 5,370,996) and stearoyl-ACP desaturases (WO 91/13972).

Of particular interest is the use of a mangosteen acyl-ACP thioesterase as a secondary gene for fatty acid content modification. As described in WO 96/36719 and WO 97/12047, a high stearate content may be obtained in seeds by expression of a mangosteen acyl-ACP thioesterase. To combine the high oleic acid trait of the 3390 plants described herein with the 5266 high stearate plants described in WO 97/12047, crosses were made between 3390-1 and 5266-35 and between 3390-1 and 5266-5. Seeds resulting from these crosses contained oil having a high stearate, low linoleic, low linolenic and high carotenoid phenotype.

Any means for producing a plant comprising the primary gene or both the primary and secondary genes are encompassed by the present invention. For example, the secondary gene of interest can be used to transform a plant at the same time as the primary gene (cotransformation), the secondary gene can be introduced into a plant which has already been transformed with the primary gene, or alternatively, transformed plants, one expressing the primary gene and one expressing the secondary

gene, can be crossed to bring the genes together in the same plant.

By combining the genes with tissue specific promoters, the carotenoid levels can be altered in particular tissues of the plant. Thus, carotenoid levels in the seed, including embryos and endosperm, can be altered by the use of seed specific transcriptional initiation regions. Such regions are disclosed, for example, in U.S. Patent No. 5,420,034, which disclosure is herein incorporated by reference.

In this manner, the transformed seed provides a factory for the production of modified oils. The modified oil may be used or alternatively, the compounds in the oils can be isolated. Thus, the present invention allows for the production of particular compounds of interest as well as speciality oils.

The primary or secondary genes encoding the enzymes of interest can be used in expression cassettes for expression in the transformed plant tissues. To alter the carotenoid or fatty acid levels in a plant of interest, the plant is transformed with at least one expression cassette comprising a transcriptional initiation region linked to a gene of interest. Such an expression cassette is provided with a plurality of restriction sites for insertion of the gene of interest to be under the transcriptional regulation of the regulatory regions.

The transcriptional initiation may be native or analogous to the host or foreign or heterologous to the host. By foreign is intended that the transcriptional initiation region is not found the wild-type host into which the transcriptional initiation region is introduced.

Of particular interest are those transcriptional initiation regions associated with storage proteins, such as napin, cruciferin, ß-conglycinin, phaseolin, or the like, and proteins involved in fatty acid biosynthesis, such as acyl carrier protein (ACP). See, U.S. Patent No. 5,420,034, herein incorporated by reference.

The transcriptional cassette will include the in 5′-3′ direction of transcription, a transcriptional and translational initiation region, a DNA sequence of interest, and a

transcriptional and translational termination region functional in plants. The termination region may be native with the transcriptional initiation region, may be native with the DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also, Guerineau et al., (1991), *Mol. Gen. Genet.*, *262*:141-144; Proudfoot, (1991), *Cell, 64*:671-674; Sanfacon et al., (1991), *Genes Dev.*, *5*:141-149; Mogen et al., (1990), *Plant Cell, 2*:1261-1272; Munroe et al., (1990), *Gene, 91*:151-158; Ballas et al., (1989), *Nucleic Acids Res., 17*:7891-7903; Joshi et al., (1987), *Nucleic Acid Res., 15*:9627-9639).

For the most part, the genes of interest of the present invention will be targeted to plastids, such as chloroplasts, for expression. In this manner, where the gene of interest is not directly inserted into the plastid, the expression cassette will additionally contain a gene encoding a transit peptide to direct the gene of interest to the plastid. Such transit peptides are known in the art. See, for example, Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep.* 9:104-126; Clark *et al.* (1989) *J. Biol. Chem. 264*:17544-17550; della-Cioppa *et al.* (1987) *Plant Physiol. 84*:965-968; Romer *et al.* (1993) *Biochem. Biophys. Res Commun. 196*:1414-1421; and, Shah *et al.* (1986) *Science 233*:478-481. Plant carotenoid genes useful in the invention may utilize native or heterologous transit peptides.

It is noted that where the gene or DNA sequence of interest is an antisense DNA, targeting to a plastid is not required.

The construct may also include any other necessary regulators such as plant translational consensus sequences (Joshi, C.P., (1987), *Nucleic Acids Research, 15*:6643-6653), introns (Luehrsen and Walbot, (1991), *Mol. Gen. Genet., 225*:81-93) and the like, operably linked to the nucleotide sequence of interest.

It may be beneficial to include 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are

known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein, O., Fuerst, T.R., and Moss, B. (1989) *PNAS USA 86*:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch Virus) (Allison et al., (1986); MDMV leader (Maize Dwarf Mosaic Virus); *Virology, 154*:9-20), and human immunoglobulin heavy-chain binding protein (BiP), (Macejak, D.G., and Sarnow, P., (1991), *Nature, 353*:90-94; untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4), (Jobling, S.A., and Gehrke, L., (1987), *Nature, 325*:622-625; tobacco mosaic virus leader (TMV), (Gallie, D.R. et al., (1989), *Molecular Biology of RNA*, pages 237-256; and maize chlorotic mottle virus leader (MCMV) (Lommel, S.A. et al., (1991), *Virology, 81*:382-385. See also, Della-Cioppa et al., (1987), *Plant Physiology, 84*:965-968.

Depending upon where the DNA sequence of interest is to be expressed, it may be desirable to synthesize the sequence with plant preferred codons, or alternatively with chloroplast preferred codons. The plant preferred codons may be determined from the codons of highest frequency in the proteins expressed in the largest amount in the particular plant species of interest. See, EPA 0359472; EPA 0385962; WO 91/16432; Perlak *et al.* (1991) *Proc. Natl. Acad. Sci. USA 88*:3324-3328; and Murray *et al.* (1989) *Nucleic Acids Research 17*: 477-498. In this manner, the nucleotide sequences can be optimized for expression in any plant. It is recognized that all or any part of the gene sequence may be optimized or synthetic. That is, synthetic or partially optimized sequences may also be used. For the construction of chloroplast preferred genes, see USPN 5,545,817.

In preparing the transcription cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate in the proper reading frame. Towards this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction

sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resection, ligation, or the like may be employed, where insertions, deletions or substitutions, *e.g.* transitions and transversions, may be involved.

The recombinant DNA molecules of the invention can be introduced into the plant cell in a number of art-recognized ways. Those skilled in the art will appreciate that the choice of method might depend on the type of plant, i.e. monocot or dicot, targeted for transformation. Suitable methods of transforming plant cells include microinjection (Crossway *et al.* (1986) *BioTechniques 4*:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA 83*:5602-5606, *Agrobacterium* mediated transformation (Hinchee *et al.* (1988) *Biotechnology 6*:915-921) and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent 4,945,050; and McCabe *et al.* (1988) *Biotechnology 6*:923-926). Also see, Weissinger *et al.* (1988) *Annual Rev. Genet. 22*:421-477; Sanford *et al.* (1987) *Particulate Science and Technology 5*:27-37(onion); Christou *et al.* (1988) *Plant Physiol. 87*:671-674(soybean); McCabe *et al.* (1988) *BioTechnology 6*:923-926 (soybean); Datta *et al.* (1990) *Biotechnology 8*:736-740(rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA, 85*:4305-4309(maize); Klein *et al.* (1988) *Biotechnology 6*:559-563 (maize); Klein *et al.* (1988) *Plant Physiol. 91*:440-444(maize); Fromm *et al.* (1990) *Biotechnology 8*:833-839; and Gordon-Kamm *et al.* (1990) *Plant Cell 2*:603-618 (maize).

Alternatively, a plant plastid can be transformed directly. Stable transformation of chloroplasts has been reported in higher plants, see, for example, SVAB *et al.* (1990) *Proc. Nat'l. Acad. Sci. USA* 87:8526-8530; SVAB & Maliga (1993) *Proc. Nat'l Acad. Sci. USA* 90:913-917; Staub & Maliga (1993) *Embo J.* 12:601-606. The method relies on particle gun delivery of DNA containing a selectable marker and targeting of the DNA to the plastid genome through homologous recombination. In such methods, plastid gene expression can be accomplished by use of a plastid gene

promoter or by trans-activation of a silent plastid-borne transgene positioned for expression from a selective promoter sequence such as that recognized by T7 RNA polymerase. The silent plastid gene is activated by expression of the specific RNA polymerase from a nuclear expression construct and targeting of the polymerase to the plastid by use of a transit peptide. Tissue-specific expression may be obtained in such a method by use of a nuclear-encoded and plastid-directed specific RNA polymerase expressed from a suitable plant tissue specific promoter. Such a system has been reported in McBride *et al.* (1994) *Proc. Natl. Acad. Sci., USA 91*:7301-7305.

The cells which have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al., Plant Cell Reports* (1986), 5:81-84. These plants may then be grown, and either pollinated with the same transformed strainer or different strains, and the resulting hybrid having the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure the desired phenotype or other property has been achieved.

As a host cell, any plant variety may be employed. Of particular interest, are plant species which provide seeds of interest. For the most part, plants will be chosen where the seed is produced in high amounts, a seed-specific product of interest is involved, or the seed or a seed part is edible. Seeds of interest include the oil seeds, such as oilseed *Brassica* seeds, cotton seeds, soybean, safflower, sunflower, coconut, palm, and the like; grain seeds, *e.g.* wheat, barley, oats, amaranth, flax, rye, triticale, rice, corn, etc.; other edible seeds or seeds with edible parts including pumpkin, squash, sesame, poppy, grape, mung beans, peanut, peas, beans, radish, alfalfa, cocoa, coffee, tree nuts such as walnuts, almonds, pecans, chick-peas etc.

In one embodiment of the invention, seed transcriptional initiation regions are used in combination with at least one carotenoid biosynthesis gene. This increases the activity of the carotenoid pathway and alters carotenoid levels in the transformed seed.

In this manner, particular genes can be selected to promote the formation of compounds of interest. Where the gene selected is an early carotenoid biosynthesis gene the transformed seed has a significant increase in carotenoid biosynthesis as the result of an icrease in the flux through the pathway. For *Brassica* seeds transformed with an early carotenoid biosynthesis gene, significant increases in the production of α-carotene, β-carotene and smaller increases in lutein in the seed oil, as well as altered oil fatty acid compositions are obtained.

Where the early carotenoid biosynthesis gene is phytoene synthase, significant increases of a particular carotenoid include those ranging from a 10 to a 50 fold increase, preferably at least a 50 to a 100 fold increase, more preferably, at least a 50 to a 200 fold increase, such as the increases seen in α-carotene and β-carotene levels. Lutein levels, in this case, are also increased, but lower increases of 1.5 - 2 fold are obtained. At the same time, total carotenoid levels may be increased at least 10 to 25 fold, preferably 25 to 60 fold, and more preferably 25 to 100 fold. Thus, a seed of the invention transformed with a phytoene synthase gene has a substantial increase in levels of α- and β-carotene and total carotenoids, as well as smaller increases in lutein and other carotenoids. In some cases, it is not possible to quantitate the fold increase in a given carotenoid compound, as the levels are too low to detect in seeds from non-transformed. In *Brassica napus*, for example, α-cryptoxanthin, lycopene, phytoene and phytofluene are all detected in various levels in seeds transformed with a *crt*B gene, but are not detectable in seeds from untransformed *Brassica napus* plants.

Where the early carotenoid biosynthesis gene is GGPP synthase, 1.5 to 2 fold increases in lutein and ß-carotene may be obtained. Lycopene is also detected in seeds from *Brassica napus* plants transformed with a *crt*E (GGPP synthase) gene. Total carotenoids in this case are also increased approximately 2 fold. Thus, also of interest as sources of carotenoids are plants which have been engineered to express increased levels of both phytoene synthase and GGPP synthase.

This metabolic energy effected by transformation with an early carotenoid gene can be funneled into a metabolic compound of choice by transformation with a second gene. As discussed above, the second gene is designed to promote the synthesis of a particular carotenoid by promoting the formation of the carotenoid of interest or alternatively by stopping the pathway to allow for the buildup of compounds. Therefore, significant amounts of carotenoids of interest can be produced in the transformed seeds of the present invention.

The seeds of the invention which have been transformed with the primary early carotenoid biosynthesis gene also provide a source for novel oil compositions. The use of phytoene synthase as the primary gene, for example, results in substantial increases in oleic acid content in seed oil. By substantial increase is intended an increase of from about 5% to about 40%, specifically from about 20% to about 40%, more specifically from about 30% to about 40%. Thus, the seeds of the invention which have been transformed with a primary early carotenoid biosynthesis gene provide a source for modified oils having a high oleic acid content. That is, carotenoid biosynthesis genes, particularly early carotenoid biosynthesis genes can be used to produce seeds having at least 70% oleic acid, on a weight percentage basis. The oleic acid content in any seed can be altered by the present methods, even those seeds having a naturally high oleic acid content. Alteration of seeds having naturally high oleic acid contents by the present methods can result in total oleic acid contents of as high as 80%.

Importantly, there is also a decrease in linoleic and linolenic acid content. By decrease in linoleic fatty acid content is intended a decrease from about 10% to about 25%, preferably about 25% to about 40%, more preferably about 35% to about 60%. By decrease in linolenic fatty acid content is intended a decrease from about 10% to about 30%, preferably about 30% to about 60%, more preferably about 50% to about 75%. Thus, the methods of the invention result in oils which are more oxidatively stable than the naturally occurring oils. The modified oils of the invention are low-

saturate, high oleic and low linolenic. Furthermore, the present invention provides oils high in monounsaturated fatty acids which are important as a dietary constituent.

Based on the methods disclosed herein, seed oil can be modified to engineer an oil with a high oleic acid content as well as a high level of a carotenoid of interest. High oleic acid and and high α- and β-carotene oils would have a longer shelf life as both the oleic acid and α- and β-carotene content would lend stability. It is also noted that such oils are more desirable as sources of carotenoids than the natural red palm oil, which oil contains high levels of saturated fatty acids.

The transformed seed of the invention can thus provide a source of carotenoid products as well as modified fatty acids. Where the intent is to produce particular carotenoid compounds of interest, methods are available in the art for the purification of the carotenoid compounds. In the same manner, methods available in the art can be utilized to produce oils purified of carotenoids. See, generally, WO 96/13149 and Favati *et al.* (1988) *J. Food Sci. 53*:1532 and the references cited therein.

In addition to altering the carotenoid levels in seeds, the tocopherol levels can be altered, preferably increased. Such seeds with increased levels of tocopherol, particularly α-tocopherol, are desirable as α-tocopherol is the most important form of the vitamin E family. Vitamin E is essential for the nutrition of humans and other animals. Evidence is available that vitamin E functions in the body in maintaining the integrity of the red blood cells, as essential in cellular respiration, is involved in the biosynthesis of DNA, and acts as an antioxidant which may have implications in protecting cells from carcinogens. Thus, seeds and oils having increased tocopherol levels are desirable. Oils having a nearly 50% increase in α-tocopherol levels are provided herein, and seed oils having even greater increases, up to 2-5 fold, are envisioned using the methods of the present invention.

The transformed seed and embryos additionally find use as screenable markers. That is, transformed seed and embryos can be visually determined and selected based

on color as a result of the increased carotenoid content. The transformed seeds or embryos display a color ranging from yellow to orange to red as a result of the increased carotenoid levels. Therefore, where plant transformation methods involve an embryonic stage, such as in transformation of cotton or soybean, the carotenoid gene can be used in plant transformation experiments as a marker gene to allow for visual selection of transformants. Likewise, segregating seed can easily be identified as described further in the following examples.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Example 1 Expression Construct and Plant Transformation

A. SSU fusions to *E. uredovora* carotenoid biosynthesis genes

(1) Phytoene Synthase

The SSU leader and *crt*B gene sequences were joined by PCR. The sequence of the SSU/*crt*B fusion is shown in Figure 1. The *crt*B gene from nucleotides 5057 to 5363 (numbering according to Misawa *et al.* (1990) *supra*) was joined to the SSU leader as follows. A *Bgl*I site was included upstream of the SSU leader start site to facilitate cloning. The thymidine nucleotide at 5057 of *crt*B was changed to an adenosine to make the first amino acid at the SSU leader/*crtB* junction a methionine, and the splice site a cys-met-asn. The native splice site for SSU is csy-met-gln. Note that Misawa *et al.* (1990) *supra*) indicates that the start site for the coding region for *crtB* is at nucleotide 5096. Thus, there are 13 amino acids upstream of the published start of the coding region for *crtB* and after the SSU splice site in the *crt*B/SSU fusion. Twelve of these amino acids are translated from *Erwinia crt*B upstream sequence and one is the added methionine. The *crtB* from 5363 (*Eco*RV) to 6009 (*Eco*RI) was then attached to the SSU-*crtB* fusion to obtain a complete SSU-*crtB* fusion construct designated pCGN3373 (Fig. 1).

(2) Phytoene Desaturase

A plasmid comprising a *E. uredovora crtI* gene fused to the transit peptide sequence of the pea Rubisco small subunit was described by Misawa *et al.* (*The Plant Journal* (1993) *4*:833-840. An approximately 2.1 kb *Xba*I/*Eco*RI fragment of this plasmid containing the SSU-*crtI* fusion and a nos 3' termination region was cloned in position for expression from a napin 5' promoter.

(3) GGPP Synthase

A similar construct containing the SSU transit fused to an *E. uredovora crtE* gene was obtained. The SSU-*crtE* fusion is present on an approximately 1.2 kb *Bgl*II/*Bam*HI fragment in pCGN3360.

B. Expression Constructs for Plant Transformation

(1) Phytoene Synthase

pCGN3373 carrying the complete SSU/*crtB* fusion was cut with *Bgl*II and *Bam*HI to excise the SSU/*crtB* fusion. The resulting fragment was ligated into the napin expression cassette in pCGN3223 at the BamHI site (see WO 94/10288 for description of napin expression cassette). The resulting construct, pCGN3389, was digested with *Hin*dIII to excise the napin 5'-SSU/*crtB*-napin 3' fragment, which was then cloned into *Hin*dIII cut pCGN1559PASS yielding pCGN3390. pCGN1559PASS is a binary vector for *Agrobacterium*-mediated transformation such as those described by McBride *et al.* (*Plant Mol. Biol.* (1990) *14*:269-276) and is prepared from pCGN1559 by substitution of the pCGN1559 linker region with a linker region containing the following restriction digestion sites: *Asp*718/*Asc*I/*Pac*I/*Xba*I/*Bam*HI/*Swa*I/*Sse*8387(*Pst*I)/*Hin*dIII. A map of pCGN3390 is provided in Figure 2A.

(2) Phytoene Desaturase

A fragment comprising a napin 5'/SSU-*crtI* fusion/nos 3' construct as described above was cloned into a binary vector for plant transformation resulting in pCGN9010. A map of pCGN9010 is provided in Figure 2C.

(3) GGPP Synthase

pCGN3360 carrying the complete SSU/*crtE* fusion was cut with *Bgl*II and *Bam*HI to excise the SSU/*crtE* fusion. The resulting 1.2 kb fragment was ligated into the napin expression cassette in pCGN3223 at the BamHI site. The resulting construct, pCGN3391, was digested with *Hin*dIII to excise the napin promoter-SSU/*crtE* napin 3' fragment, which was then cloned into *Hin*dIII cut pCGN1559PASS yielding pCGN3392. A map of pCGN3392 is provided in Figure 2B.

(4) Phytoene Synthase + Phytoene Desaturase

The napin 5'-SSU/*crtB*-napin 3' fragment from pCGN3389 and the napin 5'/SSU-*crtI* fusion/nos 3' as present in pCGN9010 were inserted into a binary vector resulting in pCGN9009, shown in Figure 2D.

(5) Antisense Epsilon Cyclase + Phytoene Synthase

Brassica napus epsilon cyclase genes are isolated by PCR using primers designed from an *Arabidopsis* epsilon cyclase gene (Cunningham FX Jr (1996) *Plant Cell 8*:1613-1626). Sequence of *B. napus* epsilon cyclase genes is provided in Figures 9 (clone 9-4) and 10 (clone 7-6). An antisense construct is prepared by cloning an*Xho*I/*Bam*HI fragment of cDNA clone 9-4 into a napin expression cassette (pCGN3223) digested with *Xho*I and *Bgl*II. The napin 5'-antisense epsilon cyclase-napin 3' fragment is cloned along with a napin 5'-SSU/*crtB*-napin 3' fragment, fragment into a binary vector for plant transformation, resulting in pCGN9002, shown in Figure 2E.

(6) Antisense Beta Cyclase + Phytoene Synthase

Brassica napus beta cyclase genes are isolated by PCR using primers designed from an *Arabidopsis* beta cyclase gene (Cunningham FX Jr (1996) *Plant Cell 8*:1613-1626). Sequence of a *B. napus* beta cyclase cDNA, 32-3, is provided in Figures 11. An antisense construct is prepared by cloning an*Xho*I fragment of the beta cyclase cDNA clone into a napin expression cassette (pCGN3223) digested with *Xho*I. A

clone containing the beta cyclase in the antisense orientation is selected. The napin 5'-antisense beta cyclase-napin 3' fragment is cloned along with a napin 5'-SSU/*crtB*-napin 3' fragment into a binary vector for plant transformation, resulting in pCGN9017, shown in Figure 2F.

C. Plant Transformation

Transformed *Brassica napus* plants containing the above described constructs are obtained as described in Radke *et al.* (*Theor. Appl. Genet.* (1988) 75:685-694 and *Plant Cell Reports* (1992) *11*:499-505).

Example 2 Analysis of Transgenic Plants

A. Visual Observations and Segregation Ratios

The napin-SSU leader/*crtB* plants in 212/86 were tagged at 21 days, 28 days and 35 days post anthesis. When the first plant, 3390-1 was harvested at 28 days, some of the seeds were obviously orange. AT 35dpa, the orange was obvious enough that a segregation ratio could be obtained. This trend of orange seeds has continued and is seen in each of the 17 lines harvested that have been obtained. A table of the segregation ratios is included below in Table 1.

TABLE 1

Generation	Plant #	Orange	Green	Ratio	Chi Square
T2	3390-1	291	88	3 to 1	0.64
T2	3390-2	150	22	No fit	
T2	3390-8	293	87	3 to 1	0.90
T2	3390-4	277	82	3 to 1	0.89
T2	3390-5	243	62	3 to 1	1.90
T2	3390-7	236	89	3 to 1	0.99
T2	3390-6	307	5	63 to 1	0.00
T2	3390-3	121	50	No fit	1.64
T2	3390-11	294	105	3 to 1	0.37

T2	3390-15	287	83	3 to 1	1.30
T2	3390-16	187	65	3 to 1	0.08
T2	3390-17	105	104	No fit	
T2	3390-12	119	28	3 to 1	2.78
T2	3390-14	283	107	3 to 1	1.23
T2	3390-19	238	94	3 to 1	1.94
T2	3390-20	251	4	63 to 1	0.00
T2	3390-27	229	4	63 to 1	0.04

B. Carotenoid Analysis of Developing Seeds

Carotenoids were extracted from seeds harvested at approximately 35 days post-anthesis as follows. Eight seed samples of orange seeds from transgenic plant 3390-1 and eight seed samples of a 212/86 variety rapeseed control plant were ground in 200µl of 70% acetone/30% methanol. The ground seed mixture was then spun in a microcentrifuge for approximately 5 minutes and the supernatant removed. Two additional 70% acetone/30% methanol extractions were conducted with the pelleted seed material and all three supernatants pooled and labeled A/M extract.

At this point in the extraction, the control seed pellets are white, whereas the seed pellets from the transgenic seeds have a yellow color. The pellets are then extracted twice with ether and the resultant supernatants pooled and labeled E extract. The A/M extract was then transferred to ether as follows. 450µl ether and 600µl of water were added to the extracts, followed by removal of the ether layers. The A/M extracts were then washed two more time with 400µl of ether, and the ether fractions from the three A/M washes pooled. The E extracts described above were washed once with 400µl of water and pooled with the A/M ether fractions. The pooled ether fractions were blown down to a volume of approximately 300µl with nitrogen gas and filtered using a syringe microfilter. The sample vials were rinsed with approximately 100µl ether and the rinse was similarly filtered and pooled with the initial filtrate, yielding total volume of approximately 150µl. A 50µl aliquot was stored at -20°C until

further analysis and the remaining 100µl sample was saponified as follows. 100µl of 10% potassium hydroxide (KOH) in methanol was added to each 100µl sample and the mixture stored in the dark at room temperature for approximately 2 hours. 400µl of water was then added to the samples and the ether phase removed. For better phase separation, saturated NaCl may be substituted for the water. The water solution was then extracted twice more with 100µl of ether and the ether samples pooled and washed with water.

The saponified samples were then analyzed by HPLC analysis on a Rainin microsorb C18 column (25cm length, 4.6mm outside diameter) at a flow rate of 1.5ml per minute. The gradient used for elution is as follows:

A = acetonitrile

B = hexane/methylene chloride (1:1)

C = methanol.

The initial solution was 70:20:10 (A:B:C). At 2.5 minutes the solution is ramped over 5 minutes to 65:25:10 (A:B:C) and held at this for 12.5 minutes. The solution is then ramped to 70:20:10 (A:B:C) over two minutes followed by a three minute delay prior to injection of the next sample. The absorbance of the eluting samples is continuously monitored at 450 and 280 nm and known chemical and biological standards were used to identify the various absorbance peaks.

In Figures 3 and 4, results of analyses of saponified samples are provided for control and pCGN3390 transformed seeds, respectively. Clear increases in the levels of α- and ß-carotene and phytoene in the transagenic plant seeds are observed, as well as smaller increases in levels of the hydroxylated carotenoid, lutein.

C. Carotenoid and Tocopherol Analysis of Mature Seeds from *crt*B Transgenic Plants

Mature 3390 T2 seed were sent to an analytical laboratory for quantitative analysis using standard HPLC methods known in the art. These results of these analysis are shown in Table 2 below. Compound levels are presented as µg/g.

Seeds designated "Maroon" were selected based on seed color. The seeds which have orange embryos appear maroon colored at maturity as opposed to the black-brown appearance of seeds from wild type plants of this cultivar. Seeds designated as "Random" were not selected for color. As 3390-1 is segregating 3 to 1 for Kan, the "Random" population includes a proportion of nulls. The maroon population contains only transgenics. Due to an effort to exclude nulls from this population, the inclusion of homozygotes may be favored.

TABLE 2

COMPOUND	CONTROL	3390-1 RANDOM	3390-1 MAROON
Lutein	7.2	18	26
Zeaxanthin	nd*	nd	nd
α-cryptoxanthin	nd	8	15
β-cryptoxanthin	nd	nd	nd
Lycopene	nd	2.3	5.1
cis-Lycopene	nd	2.9	5.4
α-carotene	0.6	124	244
β-carotene	0.9	177	338
cis-ß-carotene	0.2	12	26
Other	6	34	51
Total colored carotenoids	14.9	378.2	710.5
Phytoene	nd	62	139
Phytofluene	nd	24	54
Total all carotenoids	14.9	464.2	903.5
Alpha-tocopherol	74	93	109
Gamma-tocopherol	246	188	95
Delta-tocopherol	3	5	5

*nd = not detected

In the non-transgenic sample, "other" includes mostly very polar compounds, such as neoxanthin, violaxanthin, etc. In the transgenic sample "other" includes these and additional compounds, such as zeta-carotene, neurosporene, and mono-cyclic carotenoids.

Results of carotenoid analysis of 3390 T2 seeds from transformed plants of *B. napus* variety Quantum (SP30021) are presented in Figure 12.

Results of carotenoid analysis of 3390 T3 seeds from transformed plants of *B. napus* variety 212/86 (SP001) are presented in Figure 13.

The above results demonstrate that α- and β-carotenes levels are significantly increased in the mature seeds as the result of expression of the *crt*B gene. Generally, the overall increase in carotenoids is quite high, nearly 50 fold for colored carotenoids and up to 60 fold if phytoene and phytofluene are included. It is clear that the flux through the isoprenoid pathway has been dramatically increased. Additionally it is noted that the α-tocopherol (Vitamin E) levels are also increased by nearly 50%.

D. Germination Studies

Ten mature seeds of 3390-1 and 10 seeds of 212/86 control were planted in soil and grown in a walk-in growth chamber. The transgenics emerged 1 to 2 days later than the controls, however, all 10 seeds did germinate. The transgenics were yellowish-pink when they first emerged but greened up in one to two days. At the emergence of the first true leaf, no difference in color was observed. Plants germinated from both the transgenic and control seeds developed normally.

E. Fatty Acid Analysis

Fatty acid composition of mature seeds was determined by GC analysis of single T2 seeds harvested from trangenic plants 3390-1 and 3390-8. Single seeds from both Random (R) and Maroon (M) populations (as defined above) were analyzed and compared to seeds from a 212/86 control (SP001-1). The results of these analyses are provided in Table 3 below as weight % total fatty acids.

TABLE 3

FATTY ACID COMPOSITION OF 3390-1 AND 3390-8 LINES

SAMPLE	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
CONTROL	1.5	0	0.1	5.1	0.4	1.7	59.9	17.1	12.0	0.6	1.2	0.1	0.3
CONTROL	1.8	0	0.1	5.1	0.4	1.7	60.1	16.6	12.1	0.6	1.2	0.1	0.3
CONTROL	2.0	0	0.1	5.0	0.4	1.6	60.5	16.2	12.0	0.6	1.2	0.1	0.3
CONTROL	2.2	0	0.1	5.2	0.4	1.6	57.2	18.2	12.7	0.6	1.3	0.1	0.4
CONTROL	1.6	0	0.1	4.7	0.4	1.8	62.7	15.3	11.3	0.6	1.2	0.1	0.3
3390-1-R	2.8	0	0.1	4.8	0.5	3.6	69.9	10.6	4.8	1.2	1.1	0.0	0.6
3390-1-R*	1.5	0	0.1	4.7	0.3	1.5	58.1	19.3	12.3	0.5	1.2	0.1	0.3
3390-1-R	3.5	0	0.1	4.2	0.3	2.6	71.1	9.6	5.8	1.0	1.2	0.0	0.6
3390-1-R*	1.5	0	0.1	4.7	0.3	1.9	61.0	17.8	10.4	0.7	1.3	0.1	0.3
3390-1-R	2.2	0	0.1	4.4	0.3	3.1	73.6	8.9	4.4	1.2	1.1	0.0	0.7
3390-1-R	1.9	0	0.1	4.5	0.3	2.4	72.7	10.6	4.7	0.9	1.3	0.1	0.6
3390-1-R	2.5	0	0.1	4.2	0.3	3.4	71.7	10.0	5.1	1.1	1.0	0.0	0.6
3390-1-R	1.7	0	0.1	4.4	0.3	2.6	73.5	10.0	4.5	1.0	1.2	0.1	0.6
3390-1-R	1.9	0	0.1	4.2	0.3	2.3	72.4	9.9	6.3	0.9	1.2	0.1	0.5
3390-1-R	2.5	0	0.1	4.2	0.3	2.7	72.0	10.1	5.1	1.0	1.2	0.1	0.6
3390-1-R*	1.5	0	0.1	4.7	0.3	1.7	58.5	18.5	12.6	0.6	1.2	0.1	0.3
3390-1-R	2.8	0	0.1	4.6	0.4	3.7	71.8	9.1	4.2	1.3	1.2	0.0	0.7
3390-1-R	1.8	0	0.1	4.0	0.3	2.3	72.4	11.1	5.2	0.9	1.3	0.1	0.5
3390-1-R	1.7	0	0.1	4.4	0.3	2.7	73.9	9.9	4.2	1.0	1.2	0.1	0.6
3390-1-R	1.7	0	0.1	4.6	0.4	2.6	71.4	10.9	5.5	1.0	1.3	0.1	0.6
3390-1-R	2.7	0	0.1	4.2	0.3	2.8	72.1	9.9	5.0	1.1	1.3	0.0	0.6
3390-1-R	2.0	0	0.1	4.5	0.3	3.0	72.5	9.7	4.6	1.2	1.3	0.1	0.7
3390-1-R	1.8	0	0.1	4.9	0.4	3.4	71.8	10.4	4.2	1.2	1.2	0.0	0.7
3390-1-R*	0.9	0	0.1	4.5	0.3	1.7	55.9	18.8	15.6	0.5	1.3	0.1	0.3
3390-1-R*	1.4	0	0.1	4.8	0.4	1.7	57.1	18.0	14.4	0.6	1.2	0.1	0.3
3390-1-R*	1.4	0	0.1	4.5	0.3	1.7	57.8	18.5	13.5	0.6	1.3	0.1	0.3
3390-1-R	2.2	0	0.1	4.5	0.3	2.5	73.4	9.7	4.6	0.9	1.2	0.0	0.5
3390-1-R	1.5	0	0.1	3.8	0.3	2.7	75.9	8.1	4.6	1.0	1.4	0.0	0.6
3390-1-R	1.6	0	0.1	4.5	0.3	2.6	71.9	10.6	5.5	1.0	1.3	0.1	0.6
3390-1-R*	1.3	0	0.1	6.2	0.5	1.4	53.6	21.7	13.2	0.5	1.1	0.1	0.3
3390-1-R	2.1	0	0.1	4.3	0.3	2.4	72.3	10.7	5.1	0.9	1.2	0.0	0.6
3390-1-R*	1.3	0	0.1	5.0	0.3	1.6	57.8	18.8	13.0	0.5	1.3	0.1	0.3
3390-1-R	2.1	0	0.1	4.4	0.3	3.3	72.7	9.2	4.8	1.2	1.2	0.0	0.7
3390-1-R	1.5	0	0.1	4.5	0.3	3.3	72.6	10.1	4.6	1.2	1.1	0.1	0.7
3390-1-R*	1.2	0	0.1	4.7	0.3	1.9	59.9	17.1	12.6	0.6	1.3	0.1	0.4
3390-1-M	2.8	0	0.1	4.0	0.3	2.8	69.8	10.6	7.1	0.9	1.2	0.0	0.4
3390-1-M	2.0	0	0.1	4.9	0.4	3.3	70.3	11.1	4.9	1.2	1.2	0.1	0.7
3390-1-M	1.5	0	0.1	4.4	0.3	3.2	73.4	9.5	4.3	1.3	1.3	0.0	0.8
3390-1-M	1.5	0	0.1	4.5	0.3	2.8	72.7	10.0	5.1	1.1	1.3	0.0	0.7
3390-1-M	1.8	0	0.1	4.2	0.3	3.1	73.5	9.6	4.7	1.1	1.2	0.0	0.6
3390-1-M	1.5	0	0.1	4.7	0.3	2.9	71.6	10.7	5.1	1.1	1.2	0.1	0.7
3390-1-M	1.5	0	0.1	4.5	0.3	3.2	72.6	10.2	4.3	1.2	1.3	0.0	0.8
3390-1-M	1.8	0	0.1	4.4	0.3	2.9	72.0	10.4	5.2	1.1	1.2	0.1	0.6
3390-1-M	1.5	0	0.1	4.4	0.3	2.6	73.6	10.0	4.5	1.1	1.2	0.1	0.7
3390-1-M	2.3	0	0.1	4.3	0.3	3.0	73.0	9.7	4.5	1.1	1.2	0.0	0.6

SAMPLE	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0
3390-8-R*	1.0	0	0.1	4.9	0.3	1.6	59.2	18.9	11.9	0.5	1.2	0.1	0.3
3390-8-R	2.1	0	0.1	4.2	0.3	2.7	71.9	10.2	5.6	1.0	1.2	0.1	0.6
3390-8-R	1.5	0	0.1	4.4	0.3	2.3	72.5	10.4	5.7	0.9	1.4	0.1	0.6
3390-8-R*	1.2	0	0.1	4.9	0.3	1.7	59.7	18.2	11.6	0.6	1.3	0.1	0.4
3390-8-R*	1.5	0	0.1	4.7	0.3	1.6	58.7	18.5	12.2	0.6	1.3	0.1	0.4
3390-8-R	1.8	0	0.1	4.2	0.3	2.9	73.4	9.2	5.2	1.1	1.3	0.0	0.6
3390-8-R*	1.1	0	0.1	4.7	0.3	1.5	56.9	19.3	14.1	0.5	1.1	0.1	0.2
3390-8-R	2.2	0	0.1	4.6	0.3	3.0	71.4	10.0	5.2	1.1	1.2	0.1	0.7
3390-8-R	1.7	0	0.1	4.6	0.4	2.4	72.5	11.0	4.8	0.9	1.3	0.1	0.5
3390-8-R	2.4	0	0.1	4.7	0.3	2.9	74.0	8.4	4.0	1.1	1.2	0.0	0.7
3390-8-R	1.9	0	0.1	4.6	0.4	3.0	72.7	9.7	4.8	1.0	1.2	0.0	0.6
3390-8-R	2.0	0	0.1	4.4	0.3	2.8	73.2	9.7	4.5	1.0	1.3	0.0	0.6
3390-8-R	1.5	0	0.1	4.3	0.3	2.6	71.8	10.7	5.8	1.0	1.3	0.1	0.6
3390-8-R	1.5	0	0.1	4.4	0.3	2.7	72.6	10.5	4.9	1.0	1.3	0.1	0.6
3390-8-R	2.0	0	0.1	4.9	0.4	3.3	71.1	10.4	4.9	1.1	1.1	0.1	0.6
3390-8-R	2.1	0	0.0	4.5	0.4	3.6	73.0	8.8	4.3	1.3	1.2	0.0	0.7
3390-8-R	2.2	0	0.1	5.1	0.4	2.9	67.6	12.3	6.5	1.1	1.2	0.1	0.7
3390-8-R	1.8	0	0.1	4.2	0.3	2.6	73.5	9.9	4.8	1.0	1.3	0.1	0.6
3390-8-R	1.7	0	0.1	4.7	0.3	3.0	72.5	9.9	4.6	1.2	1.3	0.1	0.7
3390-8-R	1.7	0	0.1	4.6	0.4	2.8	73.7	9.5	4.1	1.1	1.3	0.1	0.7
3390-8-R	1.5	0	0.1	4.5	0.3	3.0	74.7	8.5	4.2	1.2	1.2	0.0	0.7
3390-8-R	1.5	0	0.1	4.4	0.4	1.9	70.0	11.8	7.2	0.8	1.4	0.1	0.5
3390-8-R	1.7	0	0.1	4.4	0.3	2.5	71.8	11.1	5.2	1.0	1.3	0.1	0.6
3390-8-R	1.4	0	0.1	4.5	0.4	2.8	73.3	9.7	4.9	1.1	1.2	0.1	0.6
3390-8-R	1.5	0	0.1	4.8	0.4	3.0	72.6	10.6	4.1	1.1	1.2	0.1	0.7
3390-8-R*	1.4	0	0.1	5.8	0.4	2.9	54.0	20.0	13.0	0.8	1.1	0.1	0.4
3390-8-R	1.4	0	0.1	4.4	0.3	2.7	71.2	10.8	6.0	1.0	1.3	0.1	0.6
3390-8-R	1.7	0	0.1	4.6	0.4	2.8	72.6	10.0	5.1	1.0	1.2	0.1	0.6
3390-8-R*	1.0	0	0.1	4.6	0.3	1.6	59.6	18.5	12.3	0.5	1.2	0.1	0.3
3390-8-R*	1.1	0	0.1	4.6	0.3	1.4	56.5	20.4	13.4	0.5	1.3	0.1	0.3
3390-8-M	1.8	0	0.1	4.7	0.4	3.3	70.1	11.1	5.5	1.2	1.1	0.1	0.7
3390-8-M	1.5	0	0.1	4.3	0.3	3.0	73.0	10.3	4.3	1.1	1.2	0.1	0.7
3390-8-M	1.9	0	0.1	4.5	0.4	3.7	73.1	8.9	4.2	1.3	1.2	0.0	0.7
3390-8-M	1.6	0	0.1	4.4	0.3	2.5	73.4	9.7	5.1	1.0	1.3	0.1	0.7
3390-8-M	1.3	0	0.1	4.4	0.3	3.0	73.7	9.6	4.4	1.1	1.3	0.0	0.7
3390-8-M	2.1	0	0.1	4.3	0.3	3.2	74.0	8.9	4.1	1.2	1.2	0.1	0.6
3390-8-M	2.1	0	0.1	3.9	0.3	1.6	71.6	11.9	5.7	0.7	1.5	0.1	0.5
3390-8-M	1.6	0	0.1	4.6	0.3	2.8	71.0	11.8	4.8	1.0	1.3	0.1	0.6
3390-8-M	2.1	0	0.1	4.8	0.4	3.2	70.3	10.7	5.2	1.2	1.2	0.1	0.7
3390-8-M	1.6	0	0.1	4.5	0.3	2.9	72.7	9.9	4.8	1.1	1.3	0.0	0.7

The above data demonstrate a substantial increase in oleic acid (18:1) in seeds from each of the transgenic lines. The increase in oleic acid is at the expense of linoleic and linolenic acids, both of which were decreased in the transgenic lines. Increases in 18:0 and 20:0 fatty acids were also observed. Based on these data, the null seeds present in the Random population can be identified, and are marked on Table 3 with an asterisk (*). All of the seeds in the Maroon populations from each transgenic line have the observed altered fatty acid compostion, confirming that the altered fatty acid composition is the result of expression of the *crt*B gene.

The trends in fatty acid composition data in the transgenic seeds which indicate positive and negative correlations of fatty acid composition changes with the observed increase in 18:1 levels are provided in Figures 5-7. The increase in 18:1 correlateswith the decreases in 18:2 and 18:3. (Figure 5). The increase in 18:1 also correlates with an increase in both 18:0 and 20:0, but little effect on 16:0 was seen (Figure 6). The increase in 18:0 also correlated with an increase in 20:0 (Figure 7).

F. Carotenoid Analysis of Mature Seeds from *crt*E Transgenic Plants

Carotenoids were analyzed in mature T2 seeds of 3392 *B. napus* plants tranformed to express the *E. uredovora crt*E gene. An approximately two fold increase in levels of lutein and ß-carotene was observed in seeds of plant 3392-SP30021-16. Lycopene was also detected in these seeds and is undetectable in seeds of untransformed control plants. Analysis of seeds from 7 additional 3392 transformants did not reveal significant increases in the carotenoid levels.

Example 3 Crosses of *crtB* Plants

A. Transgenic Oil Traits

To evaluate the high oleic trait of the napin-crtB transgenic plants in conjunction with expression of other oils traits, crosses off 3390-1-6-8 with a mangosteen thioesterase (5266) and a nutmeg thioesterase (3854; see WO 96/23892) were made. Crosses were also made with two low linoleic (LPOO4 and LP30108) varieties. Half-seed analyses of carotenoids and fatty acid composition were conducted on the segregating seeds, and the average of the half seed values are shown below in Tables 4 and 5.

Table 4

Carotenoid Levels in Half Seeds Resulting from 3390 Crosses

Cross	Lutein	Lycopene	α-Carotene	ß-Carotene	Total
F1 3390-SP001-1-6-8 x SP30021	21.6	26.2	271.5	413.1	732
F1 3390-SP001-1-6-8 x 5266-SP30021-5-26	18.0	21.7	187.9	284.1	511
F1 3390-SP001-1-6-8 x 5266-SP30021-35-2	16.2	22.1	223.0	318.4	579
F1 3390-SP001-1-6-8 x 5266-SP30021-35-12	19.5	22.9	196.8	312.8	552
F1 3390-SP001-1-6-8 x LP30108-19	23.7	22.7	213.4	355.0	614
F1 LP30108-19 x F1 3390-SP001-1-6-8	16.4	19.6	156.7	224.5	417

Table 5

Fatty Acid Composition in Half Seeds Resulting from 3390 Crosses

STRAIN_ID	%14:0	%16:0	%18:0	%18:1	%18:2	%18:3	%20:0
(3390-SP001-1-6-8 X SP30021)	0.05	3.55	1.70	74.78	11.29	5.71	0.73
(3390-SP001-1-6-8 X 5266-SP30021-35-12)	0.06	3.84	11.37	62.86	11.06	5.08	3.38
(3390-SP001-1-6-8 X 5266-SP30021-35-2)	0.06	3.68	11.27	64.80	9.81	5.16	3.04
3390-SPOO1-1-6-8 X 5266-SP30021-5-26	0.06	3.66	15.36	60.78	9.30	4.77	3.87
(3390-SP001-1-6-1 X 3854-SP30021-20-3)	2.69	9.80	3.65	64.62	9.72	4.57	1.51
(3390-SP001-1-6-1 X 3854-SP30021-20-1)	6.14	16.35	5.12	54.91	8.23	4.23	2.03
(3390-SP001-1-6-1 X 5266-LP004-2-31)	0.07	3.82	11.67	64.52	11.46	3.14	3.08
(3390-SP001-1-6-8 X LP30108-19)	0.05	3.80	1.44	73.66	14.02	3.93	0.67
(LP30108-19 X 3390-SP001-1-6-8)	0.04	3.31	1.79	79.69	9.26	2.97	0.75
SPOO1-4-10	0.07	4.44	0.99	56.06	21.79	14.31	0.44
3390-SPOO1-1-6-8	0.04	3.46	1.44	77.26	9.30	5.71	0.63

As the above results demonstrate, a dramatic increase (100 to 200 fold) in α- and β-carotene as well as a 60 fold increase in total carotenoids may be obtained by transformation of plants for expression of an early carotenoid biosynthesis gene under the regulatory control of promoter preferentially expressed in plant seed tissue. This increase in flux primes the pathway for the production of speciality products as described above, and also results in increased production of α-tocopherol (Vitamin E).

Furthermore, it is evident that the fatty acid composition can also be altered in the transgenic plant seeds. In this manner, seeds can be used to produce novel products, to provide for production of particular carotenoids, to provide high oleic oils, and the like.

All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

IN THE CLAIMS

What is claimed is:

1. A method for altering carotenoid levels in a seed from a host plant, said method comprising transforming said host plant with a construct comprising as operably linked components, a transcriptional initiation region from a gene preferentially expressed in a plant seed, a plastid transit peptide, a DNA coding sequence of at least one carotenoid biosynthesis gene, and a transcriptional termination region.

2. The method of claim 1, wherein said carotenoid levels are increased.

3. The method of claim 1, wherein said carotenoid biosynthesis gene encodes an enzyme selected from the group consisting of geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, isopentenyl diphosphate isomerase, β-carotene hydroxylase, ε-hydroxylase, lycopene ε-cyclase, lycopene β-cyclase and the astaxanthin biosynthesis enzyme encoded by crtW.

4. The method of claim 1, wherein said DNA coding sequence reduces the expression of a carotenoid biosynthesis gene native to said host plant by antisense or cosuppression.

5. The method of claim 4, wherein said carotenoid biosynthesis gene is selected from the group consisting of lycopene ε-cyclase, lycopene β-cyclase, ε-hydroxylase, β-hydroxylase, and phytoene desaturase.

6. The method of claim 2, wherein said carotenoid biosynthesis gene is not native to said host plant.

7. The method of claim 2, wherein said carotenoid biosynthesis gene is from a procaryote.

8. The method of claim 1, wherein said host plant is an oilseed *Brassica* plant.

9. The method of claim 1, wherein said transcriptional initiation region is from a gene preferentially expressed in *Brassica* seed tissue.

10. The method of claim 9, wherein said transcriptional initiation region is from a napin gene.

11. A method for increasing the carotenoid biosynthetic flux in seed from a host plant, said method comprising transforming said host plant with a construct comprising as operably linked components, a transcriptional initiation region from a gene preferentially expressed in a plant seed, a plastid transit peptide, a DNA coding sequence of a primary gene, and a transcriptional termination region, wherein said primary gene is an early carotenoid biosynthesis gene.

12. The method of claim 11, wherein said early carotenoid biosynthesis gene encodes an enzyme selected from the group consisting of geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and isopentenyl diphosphate isomerase.

13. The method of claim 12, wherein said early carotenoid biosynthesis gene encodes phytoene synthase.

14. The method of claim 11, wherein said gene is from a procaryote.

15. The method of claim 13, wherein said gene is *crt*B.

16. The method of claim 11, wherein said host plant is transformed with a second early carotenoid biosynthesis gene.

17. The method of claim 16, wherein said primary gene encodes phytoene synthase and said second early carotenoid biosynthesis gene encodes phytoene desaturase.

18. The method of claim 17, wherein said transcriptional initiation region is from a gene preferentially expressed in *Brassica* seed tissue.

19. The method of claim 17, wherein said transcriptional initiation region is from a napin gene.

20. A method for increasing α- and β-carotene in a seed from a host plant, said method comprising transforming said host plant with an expression cassette comprising as operably linked components, a transcriptional initiation region from a gene preferentially expressed in a plant seed, a plastid transit peptide, a DNA coding sequence of a primary gene, and a transcriptional termination region, wherein said primary gene is an early carotenoid biosynthesis gene selected from the group consisting of geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and isopentenyl diphosphate isomerase.

21. The method of claim 20, wherein lutein levels in said seed are increased.

22. The method of claim 20 or 21, wherein said early carotenoid biosynthesis gene encodes phytoene synthase.

23. The method of claim 20 or 21, wherein said gene is from a non-higher plant source.

24. The method of claim 22, wherein said gene is *crt*B.

25. The method of claim 20, wherein said host plant is an oilseed *Brassica* plant.

26. The method of claim 20, wherein said transcriptional initiation region is from a gene preferentially expressed in *Brassica* seed tissue.

27. The method of claim 26, wherein said transcriptional initiation region is from a napin gene.

28. A method for producing a carotenoid compound of interest in a seed, said method comprising obtaining a transformed plant which produces said seed, said plant having and expressing in its genome:

a primary gene which is operably linked to a plastid transit peptide and a transcriptional initiation region from a gene preferentially expressed in a plant seed wherein said primary gene is an early carotenoid biosynthesis gene; and,

at least one secondary gene which is operably linked to a transcriptional initiation region from a gene preferentially expressed in a plant seed, wherein said secondary gene encodes a carotenoid biosynthesis gene in the pathway for said carotenoid compound operably linked to a plastid transit peptide, or provides for transcription of a DNA sequence which results in accumulation of said carotenoid compound by inhibition of expression of an enzyme capable of modifying said carotenoid compound, wherein said inhibition is obtained by antisense or cosuppression of said enzyme.

29. The method of claim 28, wherein said early carotenoid biosynthesis gene encodes an enzyme selected from the group consisting of geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and isopentenyl diphosphate isomerase.

30. The method of claim 29, wherein said early carotenoid biosynthesis gene encodes phytoene synthase.

31. The method of claim 28, wherein said secondary gene encodes an enzyme selected from the group consisting of β-carotene hydroxylase, the astaxanthin biosynthesis enzyme encoded by *crt*W, and ε-hydroxylase, or wherein said secondary gene results in inhibition of transcription of an endogenous plant gene encoding lycopene ε-cyclase, lycopene β-cyclase or phytoene desaturase.

32. The method of claim 31, wherein said secondary gene encodes β-carotene hydroxylase.

33. The method of claim 31, wherein said secondary gene encodes the astaxanthin biosynthesis enzyme encoded by *crt*W.

34. The method of claim 31, wherein said plant expresses two secondary genes.

35. The method of claim 34 wherein said secondary genes are crtZ and crtW.

36. The method of claim 31, wherein said secondary gene results in inhibition of transcription of lycopene ε-cyclase.

37. The method of claim 34, wherein said secondary genes result in inhibition of transcription of lycopene ε-cyclase and lycopene β-cyclase.

38. The method of claim 31, wherein said secondary gene encodes ε-hydroxylase.

39. The method of claim 34, wherein said secondary genes are ε-hydroxylase and crtZ.

40. The method of claim 31, wherein said secondary gene encodes phytoene desaturase.

41. The method of claim 28, wherein said seed is *Brassica*.

42. The method of claim 27, wherein said transcriptional initiation region is from a gene preferentially expressed in *Brassica* seed tissue.

43. The method of claim 42, wherein said transcriptional initiation region is from a napin gene.

44. A method for altering fatty acid levels in a seed from a plant of interest, said method comprising transforming said plant of interest with an expression cassette comprising as operably linked components, a transcriptional initiation region from a gene preferentially expressed in a plant seed, a plastid transit peptide, a DNA coding sequence of a primary gene, and a transcriptional termination region, wherein said primary gene is an early carotenoid biosynthesis gene.

45. The method of claim 44, wherein said early carotenoid biosynthesis geneencodes an enzyme selected from the group consisting of geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and isopentenyl diphosphate isomerase.

46. The method of claim 45, wherein said early carotenoid biosynthesis gene encodes phytoene synthase.

47. The method of claim 44, wherein said plant of interest is an oilseed crop plant selected from the group consisting of oilseed *Brassica*, cotton, soybean, safflower, sunflower, palm, coconut, and corn.

48. The method of claim 47, wherein said plant is an oilseed *Brassica* plant.

49. The method of claim 46, wherein said gene is *crt*B.

50. The method of claim 44, wherein said transcriptional initiation region is from a gene preferentially expressed in *Brassica* seeds.

51. The method of claim 50, wherein said transcriptional initiation region is from a napin gene.

52. The method of claim 44 wherein said seed comprise an increase in oleic acid.

53. The method of claim 52, wherein said seed further comprises a decrease in linolenic and/or linoleic fatty acids.

54. A method for screening transformed seeds or transformed embryos, said method comprising:

transforming a plant with an expression cassette comprising as operably linked components, a transcriptional initiation region from a gene preferentially expressed in a plant seed, a transit peptide, a DNA coding sequence of at least one carotenoid biosynthesis gene, and a transcriptional termination region, wherein said early carotenoid biosynthesis gene encodes an enzyme selected from the group consisting of geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and isopentenyl diphosphate isomerase and

selecting said transformed seeds or transformed embryos exhibiting a yellow, orange or red color.

55. The method of claim 54, wherein said early carotenoid biosynthesis gene is phytoene synthase.

56. The method of claim 55 wherein said plant is an oilseed *Brassica* plant and wherein said transformed embryos exhibit an orange color.

57. A method for using a carotenoid biosynthesis gene as a marker gene in plant transformation, said method comprising:

transforming a plant a gene of interest and a carotenoid biosynthesis gene, wherein said carotenoid biosynthesis gene is operably linked to a transit peptide and a transcriptional initiation region from a gene preferentially expressed in a plant seed, and

selecting said transformed plants by screening embryos which exhibit a yellow, orange or red color or screening seeds from said transformed plants for seeds having a yellow, orange to red color.

58. The method of claim 57, wherein said carotenoid biosynthesis gene is selected from the group consisting of geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and isopentenyl diphosphate isomerase.

59. The method of claim 58 wherein said early carotenoid biosynthesis gene is phytoene synthase.

60. A transgenic plant which produces seed having altered carotenoid levels.

61. The plant of claim 60, wherein said seed produces increased levels of at least one carotenoid compound of interest, said compound of interest selected from the group consisting of α-carotene, β-carotene, lycopene, lutein, zeaxanthin, canthaxanthin, phytoene, α-cryptoxanthin, β-cryptoxanthin, ζ-carotene, phytofluene, neurosporane and astaxanthin.

62. The plant of claim 61, wherein said seed produces increased levels of α-carotene, β-carotene and lutein.

63. The plant of claim 62, wherein said seed also has altered fatty acid levels.

64. The plant of claim 63, wherein said seed have increased levels of oleic acid and decreased levels of linoleic and/or linolenic acid.

65. A transformed seed which has altered carotenoid levels.

66. The transformed seed of claim 65, wherein said seed produces increased levels of at least one carotenoid compound of interest, said compound of interest selected from the group consisting of α-carotene, β-carotene, lycopene, lutein, zeaxanthin, canthaxanthin, α-cryptoxanthin, β-cryptoxanthin, ζ-carotene, phytofluene, neurosporane, and astaxanthin.

67. The transformed seed of claim 66, wherein said seed produces increased levels of α- and β-carotene and lutein.

68. The transformed seed of claim 65, wherein said seed has altered fatty acid levels.

69. The transformed seed of claim 68, wherein said seed has increased levels of oleic acid and decreased levels of linoleic and/or linolenic acid.

70. A method for increasing tocopherol levels in a seed from a host plant, said method comprising transforming said host plant with an expression cassette comprising as operably linked components, a transcriptional initiation region from a gene preferentially expressed in a plant seed, a plastid transit peptide, a DNA coding sequence of a primary gene, and a transcriptional termination region, wherein said primary gene is an early carotenoid biosynthesis gene selected from the group consisting of geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, and isopentenyl diphosphate isomerase.

71. The method of claim 70, wherein said early carotenoid biosynthesis gene encodes phytoene synthase.

72. The method of claim 71, wherein α- tocopherol levels in said seed are increased by at least 50%.

73. The method of claim 71, wherein said gene is from a non-higher plant source.

74. The method of claim 73, wherein said gene is *crt*B.

75. The method of claim 70, wherein said host plant is an oilseed *Brassica* plant.

76. Seed produced by the method of any one of claims 1, 11, 20, 28, 44 and 70.

77. Plants produced by the method of any one of claims 1, 11, 20, 28, 44, 54, 57 and 70.

78. Oil extracted from seeds produced by the method of any one of claims 1, 11, 20, 28, 44 and 70.

79. Meal extracted from seed produced by the method of any one of claims 1, 11, 20, 28 and 70.

80. The method of any one of claims 1, 11, 20, 28 and 70 wherein said seed is from a plant selected from the group consisting of oilseed *Brassica*, cotton, soybean, safflower, sunflower, coconut, palm, wheat, barley, rice, corn, oats, amaranth, pumpkin, squash, sesame, poppy, grape, mung beans, peanut, peas, beans, radish, alfalfa, cocoa, coffee, and tree nuts.

81. The method of claim 80, wherein said seed is from an oilseed crop plant selected from the group consisting of oilseed *Brassica*, cotton, soybean, safflower, sunflower, palm, coconut, and corn.

```
BglII
|
AGATCTGCTA GAGAGCTTTG CAATTCATAC AGAAGTGAGA AAAATGGCTT CTATGATATC   60

CTCTTCCGCT GTGACAACAG TCAGCCGTGC CTCTAGGGGG CAATCCGCCG CAGTGGCTCC  120

ATTCGGCGGC CTCAAATCCA TGACTGGATT CCCAGTGAAG AAGGTCAACA CTGACATTAC  180

TTCCATTACA AGCAATGGTG GAAGAGTAAA GTGCATGAAT AATCCGTCGT TACTCAATCA  240

TGCGGTCGAA ACGATGGCAG TTGGCTCGAA AAGTTTTGCG ACAGCCTCAA AGTTATTTGA  300

TGCAAAAACC CGGCGCAGCG TACTGATGCT CTACGCCTGG TGCCGCCATT GTGACGATGT  360

TATTGACGAT CAGACGCTGG GCTTTCAGGC CCGGCAGCCT GCCTTACAAA CGCCCGAACA  420

ACGTCTGATG CAACTTGAGA TGAAAAACGCG CCAGGCCTAT GCAGGATCGC AGATGCACGA  480

ACCGGCGTTT GCGGCTTTTC AGGAAGTGGC TATGGCTCAT GATATCGCCC CGGCTTACGC  540

GTTTGATCAT CTGGAAGGCT TCGCCATGGA TGTACGCGAA GCGCAATACA GCCAACTGGA  600

TGATACGCTG CGCTATTGCT ATCACGTTGC AGGCGTTGTC GGCTTGATGA TGGCGCAAAT  660
```

FIGURE 1

```
CATGGGCGTG CGGGATAACG CCACGCTGGA CCGCGCCTGT GACCTTGGGC TGGCATTTCA   720

GTTGACCAAT ATTGCTCGCG ATATTGTGGA CGATGCGCAT GCGGGCCGCT GTTATCTGCC   780

GGCAAGCTGG CTGGAGCATG AAGGTCTGAA CAAAGAGAAT TATGCGCAC CTGAAAACCG   840

TCAGGCGCTG AGCCGTATCG CCCGTCGTTT GGTGCAGGAA GCAGAACCTT ACTATTTGTC   900

TGCCACAGCC GGCCTGGCAG GGTTGCCCCT GCGTTCCGCC TGGGCAATCG CTACGGCGAA   960

GCAGGTTTAC CGGAAAATAG GTGTCAAAGT TGAACAGGCC GGTCAGCAAG CCTGGGATCA  1020

GCGGCAGTCA ACGACCACGC CCGAAAAATT AACGCTGCTG CTGGCCGCCT CTGGTCAGGC  1080

CCTTTACTCC CGGATGCGGG CTCATCCTCC CCGCCCTGCG CATCTCTGGC AGCGCCCGCT  1140

CTAGCGCCAT GTCTTTCCCG GAGCGTCCGA ATTATCGATG AATTCGAGCT CGGTACCCGG  1200

BamHI
I
GGATCCTCTA GAGTCGACCT GCAGGCATGC AA                                 1232
```

FIGURE 1

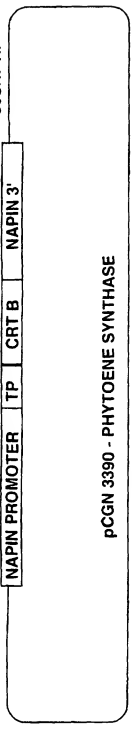


FIGURE 2A

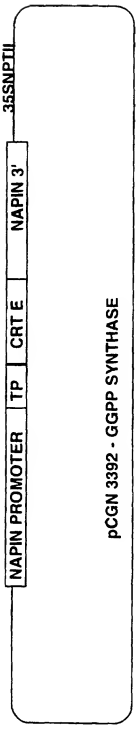


FIGURE 2B

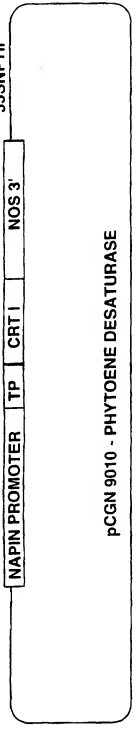


FIGURE 2C

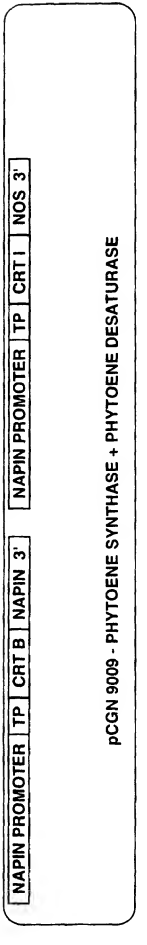


FIGURE 2D

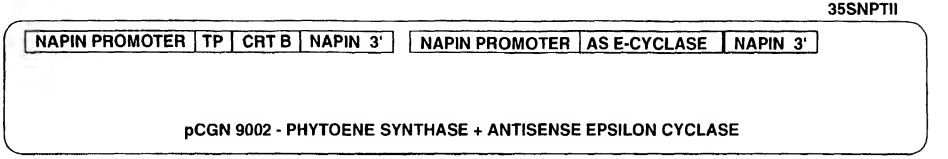


FIGURE 2E

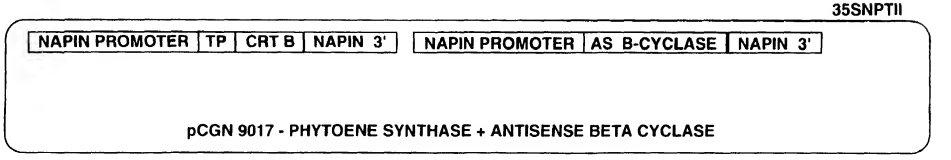


FIGURE 2F

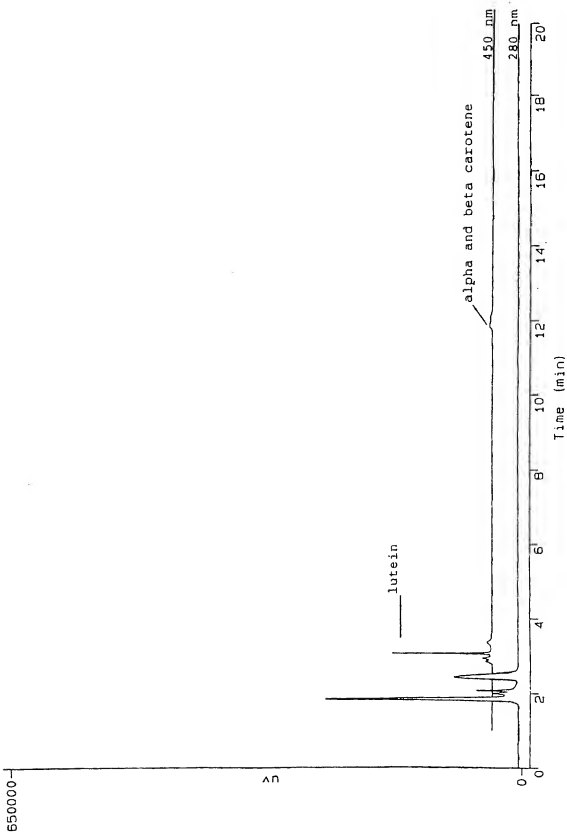


FIGURE 3

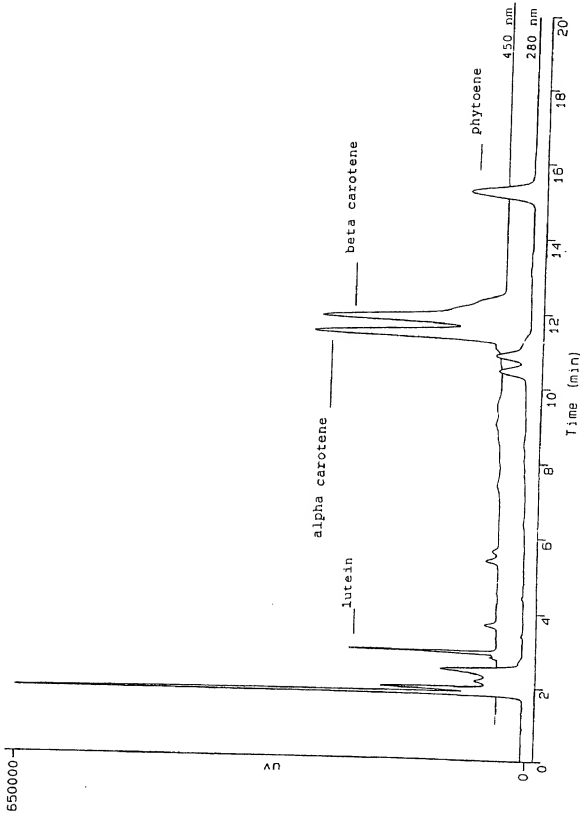


FIGURE 4

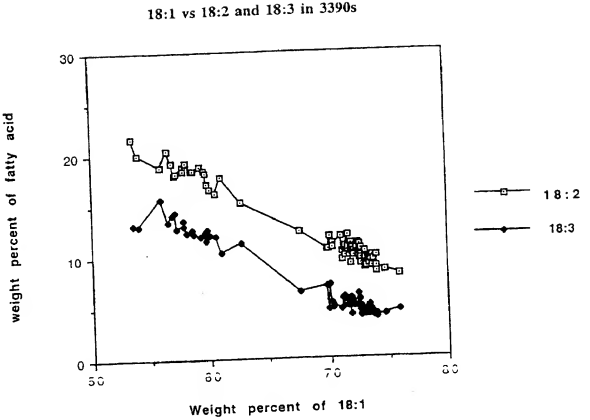

18:1 vs 18:2 and 18:3 in 3390s
weight percent of fatty acid
30
20
10
0
50
60
70
80
Weight percent of 18:1
18:2
18:3

FIGURE 5

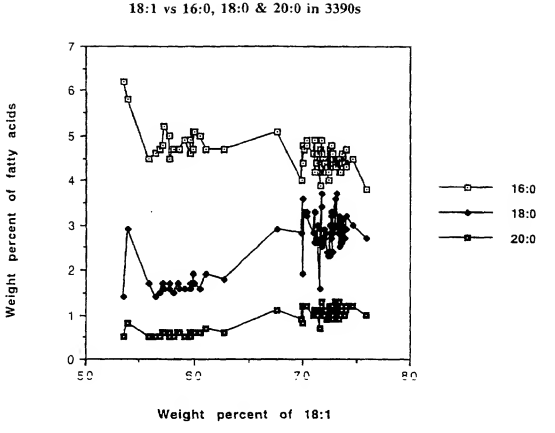

18:1 vs 16:0, 18:0 & 20:0 in 3390s
Weight percent of fatty acids
0
1
2
3
4
5
6
7
50
60
70
80
Weight percent of 18:1
16:0
18:0
20:0

FIGURE 6

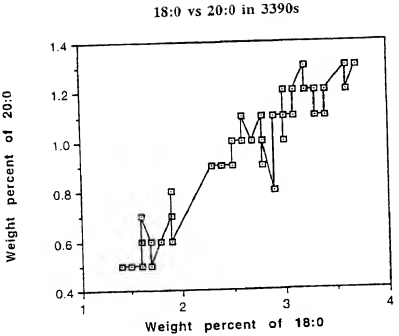

18:0 vs 20:0 in 3390s
Weight percent of 20:0
1.4
1.2
1.0
0.8
0.6
0.4
1
2
3
4
Weight percent of 18:0

FIGURE 7

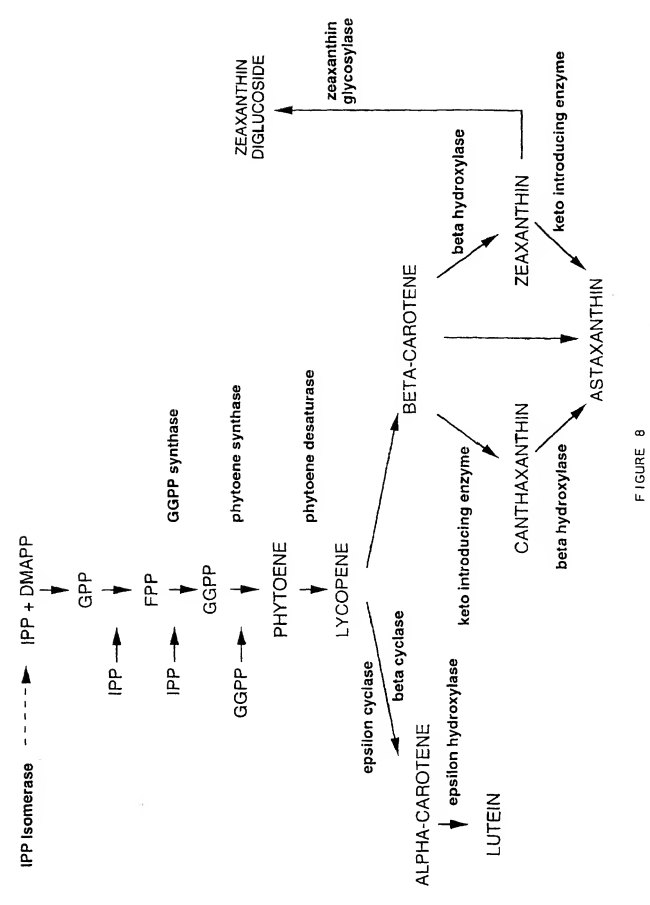
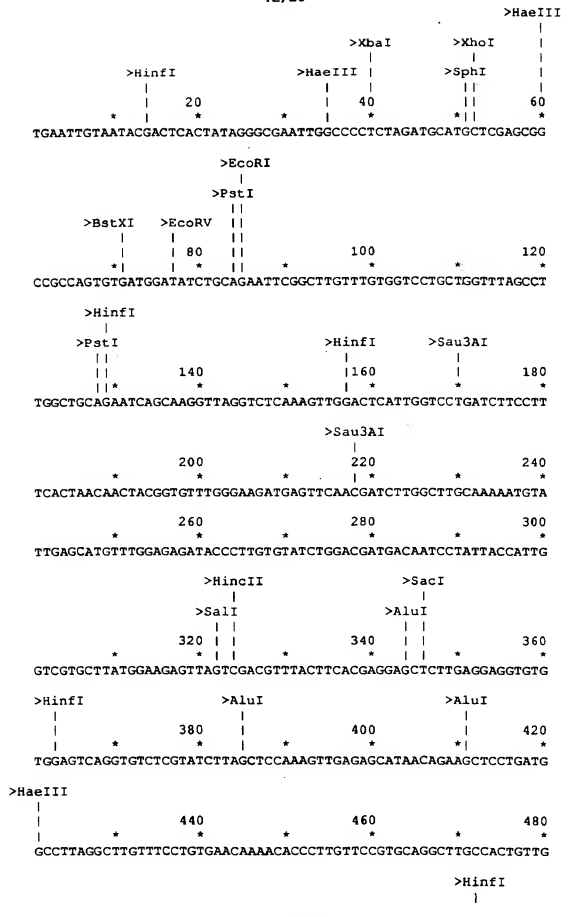


FIGURE 8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>XbaI
>XhoI
>HinfI
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>SphI
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40
60
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>EcoRI
>PstI
>BstXI
>EcoRV
80
100
120
CCGCCAGTGTGATGGATATCTGCAGAATTCGGCTTGTTTGTGGTCCTGCTGGTTTAGCCT
>HinfI
>PstI
>HinfI
>Sau3AI
140
160
180
TGGCTGCAGAATCAGCAAGGTTAGGTCTCAAAGTTGGACTCATTGGTCCTGATCTTCCTT
>Sau3AI
200
220
240
TCACTAACAACTACGGTGTTTGGGAAGATGAGTTCAACGATCTTGGCTTGCAAAAATGTA
260
280
300
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>HincII
>SacI
>SalI
>AluI
320
340
360
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>HinfI
>AluI
>AluI
380
400
420
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>HaeIII
440
460
480
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>HinfI

FIGURE 9
1/2

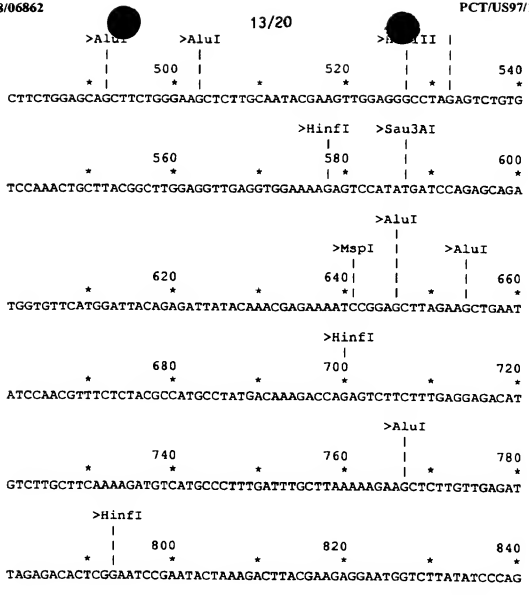

FIGURE 9

```
     >HinfI         >HaeIII >XbaI   >XhoI >SphI  >HaeIII  >BstXI  >EcoRV  >PstI >EcoRI
        10         20         30         40         50         60         70         80         90        100        110        120
GTGAATTGTA ATACGACTCA CTATAGGGCG AATTGGCCCT TCTAGATGCA TGCTCGAGCG GCCGCCAGTG TGATGGATAT CTGCAGAATT CGGCTTGTTT GTGGTCCTGC TGGTTTAGCC

>BglI  >HinfI  >AluI                                                                                  >Sau3AI
       130        140        150        160        170        180        190        200        210        220        230        240
TTGGCGGCTG AATCAGCTAA GTTAGGACTT AAAGTTGGAC TGATTGGTCC TGACCTTCCT TTCACTAACA ACTACGGTGT TTGGGAAGAT GAGTTCAACG ATCTTGGCTT GCAAAAATGT

                                                                                 >SalI >HincII
       250        260        270        280        290        300        310        320        330        340        350        360
ATTGAGCATG TTTGGAGAGA TACCCTTGTG TATCTGGACG ATGACAATCC TATTACCATT GGTCGTGCTT ATGGAAGAGT TAGTCGACGT TTACTTCACG AGGAGTTCTT GAGGAGGTGT

>HinfI                >AluI                        >AluI    >HaeIII
       370        380        390        400        410        420        430        440        450        460        470        480
CTGGAGTCAG GTGTCTCGTA TCTTAGCTCC AAAGTTGAGA GCATAACAGA AGCTCCTGAT GGCCTTAGGC TTGTTTCCTG TGAACAAAAC ACCCTTGTTC CGTGCAGGCT TGCCACTGTT

          >AluI      >AluI                >HaeIII  >HinfI                                        >HinfI    >Sau3AI
       490        500        510        520        530        540        550        560        570        580        590        600
GCTTCTGGAG CAGCTTCTGG GAAGCTCTTG CAATACGAAG TTGGAGGGCC TAGAGTCTGT GTCCAAACTG CTTACGGCTT GGAGGTTGAG GTGGAAAAGA GTCCATATGA TCCAGAGCAG

                                       >MspI >AluI   >AluI                                         >HinfI
       610        620        630        640        650        660        670        680        690        700        710        720
ATGGTGTTCA TGGATTACAG AGATTATACA AACGAGAAAA TCCGGAGCTT AGAAGCTGAA TATCCAACGT TTCTCTACGC CATGCCTATG ACAAAGACCA GAGTCTTCTT TGAGGAGACA

                                                 >AluI                      >HinfI
       730        740        750        760        770        780        790        800        810        820        830        840
TGTCTTGCTT CAAAAGATGT CATGCCCTTT GATTTGCTTA AAAAGAAGCT CTTGTTGAGA TTAGAGACAC TCGGAATCCG AATACTAAAG ACTTACGAAG AGGAATGGTC TTATATCCCA

                                 >HinfI              >PstI >AluI                                   >Sau3AI >BglII  >AluI
       850        860        870        880        890        900        910        920        930        940        950        960
GTAGGTGGTT CCTTGCCAAA CACGGAACAA AAGAATCTCG CCTTTGGTGC TGCAGCTAGC ATGGTTCATC CTGCAACAGG CTATTCAGTT GTGAGATCTT TGTCTGAAGC TCCAAAATAC
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FIGURE 10 1/2

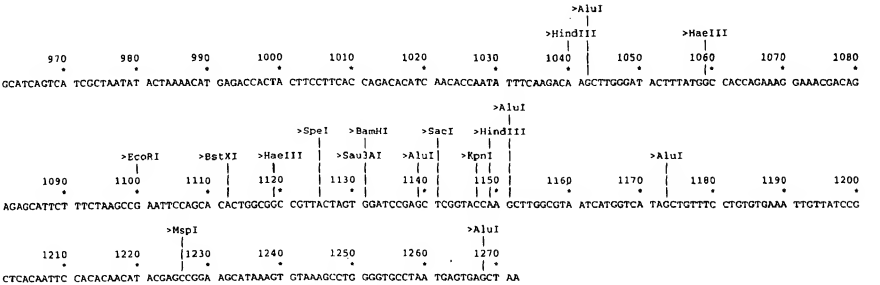

FIGURE 10 2/2

60
*
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120
*
AAATTGTTGA TCATCTTAGC AAGAGGAACA GTTCCCTTCG TCATGATCTC CAACCTCGAG

180
*
GTATTAGAAG CATGCGAGAA GAGCGACAGC CCGAAGAACA CCAGGTCCGG GAGAAACAGC

240
*
CTCGACGACA AGAAACCATG CCAGTAACGC GGTTCCAGGT CAAAGAACGC ATCAAAGAAC

300
*
CTCCTAGTAG CATCCAAATC AAGCTTCAGC AAAATATCCA TCCCAAAACA GAAGAACTCC

360
*
CTCTGTCTCC GCCTCTCAAT AGGCCACAAG TCTCTCCACA CCTCAGCCGA GAGCTCATCT

420
*
CCTCTCAAGC CGTTGTTGTT ACCACCACCA AGGTACCGCA CTATAGCGTT TGCAACTATC

480
*
GGAGCAGCTG CAAGAGTCCT AGCAACCATG TAACCAGTCG AAGGATGAAC CATCCCCGCC

540
*
GTACCGCCAA TGCCAACAAC TCTTTGAGGC AAGACCGGTA AAGGACCTCC CATAGGGATC

FIGURE 11
1/3

```
                                                                  600
                                                                    *
ACACAACGCT CGTCTTCCTC AATCCGCTTC ACGTTGATCC CCAAATGTTT CAGCCTCGCA
                                                                  660
                                                                    *
ACCATCCTCT CTTGGATATC TTCCATCTTC AGACCCGGCC TAGCCACAAG AGACGTCTCT
                                                                  720
                                                                    *
TCAAGAAAGA TCCTGTTGGA AGAAAACGGC ATCGCGTACA GGAACGTAGG GATCTTGCTG
                                                                  780
                                                                    *
TTCCGCTCTT TAACCTCAGG GTACGCGTCA AGATGCTTAT CTCTCCAGTC CATGAACACC
                                                                  840
                                                                    *
ATCTTATCCA CATCAAACGG GTGACCATCG ACCTCAGCAA TGATACCATA AGCTACTTGA
                                                                  900
                                                                    *
TACCCAGGGT TATAAGGCTT ATCATACTGA ACCAAGCATC TTGAAAAACC AGTAGCGTCG
                                                                  960
                                                                    *
AGAACAACAG AAGCCTGAAT CTTCACACCG TCACTGCAGA CAACAGTGGA GTTAACCTCC
                                                                 1020
                                                                    *
TCGTGAACCA CGTCAGTGAC TTTAGCCTGA TGGAATCTAA CACCGTTGGT GATGCACTTC
                                                                 1080
                                                                    *
TGAAGCATCT TGGATTTGAG CTGTTTACGG TTCACTCTCC CGTAAGGCCG GGACAGGTCC
```

FIGURE 11
2/3

```
                                                                  1140
                                                                   *
TTTTCGGAGC CGTCGTTGAT GTAGACGACG GCGCCGGACC AGGTGGTGTC GAGGCAGTCT

                                                                  1200
                                                                   *
AGCAAGTCCA TGGCTTCGAA CTCGTCAACC CAAACTCCGT AGTTGTTAGG CCAAATGAGT

                                                                  1260
                                                                   *
TTGGGGGAAG GATCGATGGA GCAGACAGAG AGTCCAGCTT CGGAGACTTG CTGAGCCACG

                                                                  1320
                                                                   *
GCTAAACCAG CGGGGCCGCC GCCAACGATA GCTAGATCAA CAACTTTGTT CAGGGAAGTG

                                                                  1380
                                                                   *
TCGTTTAAAG GAAGGTCCAA GTCGAGATTC TCCTTCTTGG TTTCAGGAAC AAGATCCAAA

                                                                  1440
                                                                   *
AGAGCACTAC TAGCACTAGT GATACTACTA CCGATTCTGA TTGCTCTTTT CTTCAAACCA

                                                                  1500
                                                                   *
AGCTTAACCC TTGAAGGATT TGGACTTAAT CTCTCGAACC CATGAAACTG AGGGATGAAA

                                                                  1560
                                                                   *
AACTCGAGCT TGTTGGGTGT TTTCAACAGA GTATCCATCG AATTCTGCAG ATATCCATCA

CACTGGCGGC CGCTCGAGCA TGCATCTAGA
```

FIGURE 11
3/3

	Segregation	Carotenoid concentration (µg/gFW)				
Sample ID #	ratio	Lutein	Lycopene	α-Carotene	β-Carotene	Total
SP30021 control 1		24.4	ND	ND	1.9	26.3
SP30021 control 2		34.0	ND	ND	4.9	38.9
T2 3390-SP30021-1	3:1	33.5	6.1	229.0	385.7	654.3
T2 3390-SP30021-2	15:1	50.4	6.2	372.4	721.4	1150.4
T2 3390-SP30021-3	no fit	45.8	3.9	352.9	580.9	983.5
T2 3390-SP30021-4	3:1	31.0	4.9	306.1	463.3	805.3
T2 3390-SP30021-5	3:1	36.8	10.5	370.6	659.4	1077.3*
T2 3390-SP30021-6	15:1	46.9	9.1	445.1	797.0	1298.1
T2 3390-SP30021-7	15:1	51.2	7.4	494.9	941.4	1494.9
T2 3390-SP30021-8	no fit	41.9	11.3	468.4	904.3	1425.9
T2 3390-SP30021-9	>63:1	68.4	11.9	394.2	949.2	1423.7
T2 3390-SP30021-10	null	51.6	ND	12.6	22.8	87.0
T2 3390-SP30021-11	3:1	52.2	9.5	409.8	714.5	1186.0*
T2 3390-SP30021-12*	3:1	48.0	10.2	400.0	738.8	1197.0*
T2 3390-SP30021-13	3:1	66.1	3.9	98.1	216.0	384.1
T2 3390-SP30021-14	3:1	49.1	8.9	320.0	611.6	989.6
T2 3390-SP30021-15	null	27.0	ND	ND	1.2	28.2
T2 3390-SP30021-16	3:1	55.6	6.4	283.1	527.4	872.5
T2 3390-SP30021-17	3:1	53.0	9.1	324.9	614.3	1001.3
T2 3390-SP30021-18	>63:1	49.6	8.1	449.0	759.3	1266.0
T2 3390-SP30021-19	3:1	62.2	7.6	346.1	613.2	1029.1
T2 3390-SP30021-20	3:1	52.1	6.3	285.0	544.9	888.3
T2 3390-SP30021-21	3:1	56.2	4.1	187.9	334.2	582.4
T2 3390-SP30021-22	null	43.1	ND	ND	4.9	48.0
T2 3390-SP30021-23	3:1	71.0	10.9	358.6	693.9	1134.4*
T2 3390-SP30021-24	no fit	53.9	7.3	272.1	520.4	853.7
T2 3390-SP30021-25	3:1	31.9	12.2	309.1	580.9	934.1
T2 3390-SP30021-26*	3:1	34.3	9.3	311.2	584.4	939.2*
T2 3390-SP30021-27	3:1	52.6	9.8	299.8	686.3	1048.5*
T2 3390-SP30021-28	no fit	68.4	10.0	446.3	907.7	1432.4
T2 3390-SP30021-29	>63:1	85.1	8.5	459.4	822.9	1375.9
T2 3390-SP30021-30	3:1	63.7	5.8	356.9	598.4	1024.8
T2 3390-SP30021-31	3:1	76.0	7.3	302.5	527.1	912.9
T2 3390-SP30021-32	null	51.8	2.3	31.4	55.0	140.5
T2 3390-SP30021-33	3:1	36.3	8.9	283.1	546.9	875.2
T2 3390-SP30021-34	>63:1	86.9	12.1	502.3	808.3	1409.6
T2 3390-SP30021-35	3:1	39.3	8.1	224.5	461.0	732.9
T2 3390-SP30021-36	15:1	55.5	11.0	538.5	829.9	1434.9
T2 3390-SP30021-37*	3:1	50.3	10.0	291.1	625.9	977.3*
T2 3390-SP30021-38	3:1	70.5	8.1	309.0	576.1	963.7
T2 3390-SP30021-39	null	37.3	ND	ND	3.6	40.9
T2 3390-SP30021-40	3:1	37.5	1.8	251.1	505.2	796.0
T2 3390-SP30021-41	3:1	47.5	8.4	414.1	719.3	1189.3*
T2 3390-SP30021-42	3:1	42.6	5.1	230.3	352.9	630.9
T2 3390-SP30021-43	no fit	83.3	5.6	128.4	219.8	437.9
T2 3390-SP30021-46	3:1	21.6	1.4	211.2	368.3	602.5
T2 3390-SP30021-47	3:1	79.1	3.7	312.5	570.5	965.8
T2 3390-SP30021-48	3:1	45.3	3.0	225.2	401.5	675.0
T2 3390-SP30021-49	15:1	28.3	1.6	346.0	677.2	1053.1
T4 3390-SP001-1-6-13	Homo	52.4	1.5	439.5	669.3	1162.7

FIGURE 12

Sample ID #	Segregation status	Carotenoid concentration (μg/gFW) Lutein	Lycopene	α-Carotene	β-Carotene	Total
T3 3390-SP001-4-12	Homo	43.9	17.2	282.1	636.8	980.0
T3 3390-SP001-5-7	Het	50.7	6.3	190.6	386.8	634.4
T3 3390-SP001-5-12	Homo	45.5	19.5	255.9	633.4	954.3
T3 3390-SP001-11-6	Homo	46.5	12.8	372.2	538.4	969.9
T3 3390-SP001-11-9	Homo	54.0	10.2	406.0	556.0	1026.2
T3 3390-SP001-14-2	Homo	59.7	12.5	342.4	764.0	1178.6
T3 3390-SP001-14-6	Homo	66.3	12.9	431.0	673.9	1184.1
T3 3390-SP001-15-9	Homo	30.8	14.3	271.8	559.8	876.7
T3 3390-SP001-15-12	Homo	39.6	13.1	241.7	649.1	943.5
T3 3390-SP001-16-3	Homo	49.9	17.1	230.2	519.7	816.9
T3 3390-SP001-16-6	Homo	35.5	21.1	263.8	547.7	868.1
T3 3390-SP001-35-2	Het	37.6	7.2	125.4	313.9	484.1
T3 3390-SP001-35-10	Homo	43.7	16.6	234.7	503.9	798.9
T3 3390-SP001-35-12	Homo	50.2	21.3	361.7	695.7	1128.9
T3 3390-SP001-8-3	Het	41.4	9.9	178.2	434.4	663.9
T3 3390-SP001-8-9	Homo	39.1	18.2	309.3	505.0	871.6
T3 3390-SP001-8-11	Homo	35.9	19.6	260.7	580.4	896.6
T3 3390-SP001-18-8	Het	29.2	12.2	112.1	247.6	441.1
T3 3390-SP001-16-10	Het	38.0	14.6	248.2	486.3	787.1
T4 3390-SP001-1-6-1	Homo	27.8	20.5	248.7	379.1	676.1
T4 3390-SP001-1-6-8	Homo	38.5	16.8	304.1	383.9	743.3
VAR SP001-4-5		54.2	ND	ND	5.8	60.0
VAR SP001-4-6		51.2	ND	ND	7.0	58.2
VAR SP001-4-10		30.2	ND	ND	ND	30.2

FIGURE 13

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tional Application No
T/US 97/14035

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/52 C12P17/06 C12P23/00 C12P7/64
C11B1/00 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12P C11B A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	WO 96 13149 A (AMOCO CORP) 9 May 1996 see page 8, line 27 - page 9, line 7 see page 18, line 9 - line 14	1-3,6,7, 11-15, 20-24, 60-62, 65,66, 77,80,81
Y	see page 36, line 19 - page 37, line 6	3,12,20
A	see page 68, line 25 - page 69, line 14	54,57
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
15 January 1998	30/01/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

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